Genetic analysis of the metabolic regulation of senescence in Arabidopsis thaliana

By

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Declaration:

I hereby confirm that the work presented in this thesis is my own work.

Sarah Purdy, 19th December 2006

Abstract

Leaf senescence is a sensitive and dynamic process that is both genetically and environmentally controlled. Environmentally, factors such as daylength and nitrogen availability are understood to affect the timing of senescence, but the underlying genetic factors that initiate the processes that result in leaf death remain elusive. Sugars and nitrogen can act at the molecular level as signalling molecules which result in altered gene expression. Sugars were found to accumulate in senescing leaves suggesting that leaf senescence is not caused by sugar starvation. Nitrogen decreases in senescing leaves as it is exported to young leaves and the developing fruits. This study presents evidence that the alteration in the ratio between these two essential nutrients may act as the instigating signal for leaf senescence. On medium containing 2% glucose in combination with low nitrogen supply senescence was accelerated. The expression of a number of genes, functional during developmental senescence was altered by the addition of glucose. The expression of *SAG12*, a senescence specific gene, confirmed that accelerated senescence was not caused by stress and also that glucose-induced senescence was representative of developmental senescence. Quantitative trait loci analysis was carried out on the Bay-0 x Shahdara population in response to treatment with low nitrogen plus 2% glucose. The decline in maximum photosynthetic efficiency (F_v/F_m) was monitored by chlorophyll fluorescence imaging to determine the extent of senescence of the whole rosette. Using F_v/F_m as quantitative trait, two new loci, on Chromosomes II and IV, were mapped that regulate glucose-induced senescence. The use of near isogenic lines confirmed the locus on Chromosome IV. The study of flowering uncovered a strong link between flowering time and senescence in the RIL population. In one RIL, 310, flowering was severely retarded and glucose did not induce senescence. This was confirmed by the absence of *SAG12* expression in glucose-treated plants. Of particular interest was that *FRIGIDA*, a gene involved in vernalization-dependent flowering, mapped to the QTL on chromosome IV. This gene was subsequently nominated as a candidate gene.

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To

Mum and Dad

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List of abbreviations

ABA	Abscisic acid
ANOVA	Analysis of variance
aov	analysis of variance
Asn	Asparagine
Asp	Aspartate
CIM	Composite interval mapping
DAP	Days after planting
DET	Detached leaves
DIS	Dark induced senescence
Fd-GOGAT	Ferredoxin-dependent glutamate synthase
Fru	Fructose
Fv/Fm	Maximum photosnthetic efficiency
GABA	GABA aminobutyric acid
GDH	Glutamate dehydrogenase
GLISEN	Glucose-induced senescence
Gln	Glutamine
Glu	Glutamate
GS	Glutamine synthetase
HIF	Heterozygous inbred family
HN	High nitrogen
HNG	High nitrogen plus 2% glucose
HXK	Hexokinase
Ile	Isoleucine
Leu	Leucine
LHCPII	Light harvesting complex II
lm	linear mixed
LN	Low nitrogen
LNG	Low nitrogen plus 2% glucose
LNM	Low nitrogen plus mannitol
LNS	Low nitrogen plus sorbitol
LOD	Log of odds
MLP	Membrane lipid peroxidation
NCC	Non fluorescence chlorophyll catabolites
NIL	Near isogenic line
NPQ	Non photochemical quenching
NR	Nitrate reductase
NS	Natural senescence
OPDA	12-Oxophytodienoic acid
PAM	Pulse-amplitude modulated
PaO	Pheophorbide a oxygenase
PCD	Programmed cell death
PCR	Polymerase chain reaction
PSII	Photosystem II
QTL	Quantitative trait loci

RCCR	Red chlorophyll catabolite reductase
RIL	Recombinant inbred line
RLK	Receptor-like kinase
ROS	Reacive oxygen species
RP	Percentage reddening
RT-PCR	Reverse transcriptase polymerase chain reaction
RV	Visual reddening
SAG	Senescence associated gene
SIM	Simple interval mapping
Suc	Sucrose
YP	Percentage yellowing
YV	Visual yellowing

Chapter 1

Introduction

1.1 Senescence

1.1.1. An overview

Leaf senescence is a process familiar to anyone living in a temperate climate as it is the process by which deciduous trees produce their spectacular autumn colours. In the life of annual plants, senescence marks the final developmental stage as it is the process by which the photosynthetic apparatus is disassembled and stored nutrients are remobilized to the developing reproductive structures. The final stage of senescence results in the death of the leaf. The developing seeds are dependent on receiving remobilized nutrients for their formation and viability and so the ordering and progression of senescence is essential. A delicate equilibrium must exist to ensure that adequate degradation occurs to provide essential nutrients during reproductive development but that enough photosynthetic structures are retained until the very late stages to produce adequate energy for the necessary remobilisation to occur. Furthermore, the degradation of the nutrient-storing chloroplast produces hazards in the form of reactive oxygen species (ROS). This occurs when chlorophyll is still present, but the electron transport chain is disrupted giving rise to the production reactive oxygen species such as singlet oxygen ($^1O_2$) or superoxide ($O_2^{\cdot-}$) that may cause lipid peroxidation and, ultimately, premature death. The necessity for

meticulous organisation and rapid response to changing environmental stimuli thus becomes apparent.

1.1.2 Chlorophyll breakdown

The first visible symptom of leaf senescence is yellowing which is caused by chlorophyll degradation. By this time, however, the majority of senescence processes have already taken place (Buchanan-Wollaston et al. 2003). The regimentation of the process is essential to ensure that damage or premature death from the build up of reactive oxygen species does not occur. The degradation of chlorophyll can be more accurately described as "chlorophyll detoxification" (Hörtensteiner, 2004) as the nitrogen contained in the end product, non-fluorescent chlorophyll catabolites (NCCs), is not recycled (Buchanan-Wollaston et al. 2003) and thus the sole purpose of the breakdown is to neutralise the reactive intermediates.

Chlorophyll detoxification can be divided into an "early" and "late" stage (Takamiya, et al. 2000; Hörtensteiner, 2006) and the division between the two is defined by the cleaving of the tetrapyrrole macrocycle ring by an oxygenase (Takamiya et al. 2000). The early stage comprises a series of processes that prepare the macrocycle for cleaving. These include "dephytylation" which is the hydrolysis of the phytol residue in ring IV, the release of magnesium from the macrocycle by $2H^+$ and modifications to the side chain of the macrocycle (Takamiya et al. 2000). The first stage is the dephytylation which is catalysed by chlorophyll-chlorophyllido hydrolase (Chlase). This enzyme is located on the plastid envelope (Matile et al. 1999) which makes it spatially separated from chlorophyll-protein complex located in the thylakoid. It has

therefore been proposed that a chlorophyll carrier protein, possibly plastoglobuli, may exist to "shuttle" chlorophyll to the plastid envelope for dephytylation (Takamiya et al. 2000). The second reaction to occur in the early stage of chlorophyll detoxification results in the release of Mg^{2+} from the tetrapyrrole macrocycle (Takamiya et al. 2000). The release of Mg^{2+} occurs in exchange for $2H^{+}$ (Matile et al. 1999) to yield pheophorbide *a*, the cleavage of which marks the transition into the late stage of chlorophyll catabolism.

The two enzymes that catalyse the opening of the tetrapyrrole macrocycle are pheophorbide *a* oxygenase (PaO) and red chlorophyll catabolite reductase (RCCR). The first product generated by the cleaving is a colourless intermediate called primary fluorescent chlorophyll catabolite (pFCC) (Hörtensteiner, 2004). The reaction requires a protein component from both the membrane and stroma of the senescing chloroplast (gerontoplast) and the addition of two oxygen and four hydrogen atoms to pheophorbide *a* (Matile et al. 1999). PaO catalyses the oxygenolysis of pheophorbide *a* which results in the production of red billin RCC. The action of the second enzyme, RCCR then reduces this red billin RCC to yield pFCC (Matile et al. 1999). The PaO enzyme requires oxygen to incorporate into the pheophorbide *a*, but RCCR is sensitive to oxygen (Hörtensteiner, 2004). Conversely, RCCR requires reduced ferredoxin and anaerobiosis (Takamiya et al. 2000). This suggests that pFCC conversion is localised to the periphery of the stromal envelope (Hörtensteiner, 2004) as this allows both an anaerobic micro-environment for the reduction to occur and the availability of molecular oxygen for the oxygenation to occur (Takamiya et al. 2000).

The pFCCs are converted to fluorescent chlorophyll catabolites through processes involving demethylation and hydroxylation, although the specific enzymes have not yet been identified (Takamiya et al. 2000). A possible candidate for the demethylation is a methylesterase, as has been identified in *Chenopodium album* (Matile et al. 1999). The final stage of chlorophyll detoxification is a non-enzymatic process which occurs in the central vacuole and involves rearrangement of the double bonds in pyrrole D and the γ methine bridge (Takamiya et al. 2000).

The degradation pathway outlined above is only functional in the detoxification of chlorophyll *a* as PaO is specific for chlorophyll *a* only (Hörtensteiner, 2004). Furthermore, no final degradation products of chlorophyll *b* are found in higher plants (Takamiya et al. 2000). The fate of chlorophyll *b*, therefore, relies on its conversion to chlorophyll *a* (Scheumann et al. 1998). The reduction of chlorophyll *b* to chlorophyll *a* requires phytyldiphosphate and reduced ferredoxin to convert the formyl group of chlorophyll *b* to the characteristic methyl group of chlorophyll *a* (Scheumann et al. 1998) for detoxification to occur.

1.1.3 Dismantling of the chloroplast

The principal purpose of performing senescence in such a structured and organised manner is to ensure that stored nutrients are recycled. It has been estimated that about 75% of mesophyll nitrogen is stored in the chloroplast proteins (Hörtensteiner and Feller, 2002) and of this, around 80% will be remobilized during the senescence process. Large proteins such as Rubisco contain the majority of the bound nitrogen and the family of stromal enzymes to which it belongs, are dissembled early in

senescence resulting in the characteristic drop in photosynthesis (Hörtensteiner and Feller, 2002).

The degradation of chloroplast-bound proteins to yield free amino acids relies on the action of endopeptidases and exopeptidases. The former of these two enzymes initiates the catabolism of a protein by catalysing the first cleavage of the peptide bonds (Hörtensteiner and Feller, 2002). The action of metalloendopeptidases and aminopeptidases has been suggested to play a role in the complete degradation of proteins by acting on the peptides produced by the action of endopeptidases (Hörtensteiner and Feller, 2002). Other suggested routes of proteolysis, such as the Clp family of proteases and reactive oxygen species (ROS), have been received with a degree of scepticism (Hörtensteiner and Feller, 2002; Buchanan-Wollaston et al. 2003).

The Clp family of proteases consists of a regulatory ATPase/chaperone and proteolytic subunits (Schelin et al. 2002). The chaperone subunits regulate a diverse range of processes including protein folding, stabilisation, renaturation and resolubilisation and, together with the ATP-dependent protease, they monitor the state of cellular proteins and remove harmful polypeptides formed, for example, by misfolding (Schelin et al. 2002). The role that Clp proteases play in plant development appears to be more closely associated with chloroplast development and maintenance than senescence, however, as yet no definitive role of this family in the regulation of senescence has been established (Hörtensteiner and Feller, 2002; Buchanan-Wollaston et al. 2003).

Reactive oxygen species are known to accumulate during senescence and it has been reported that they may initiate the degradation of Rubisco (Ishida et al. 1999) and upregulate the metallothionein gene, *LSC54* (Navabpour et al. 2003). In this latter study the authors also reported that the levels of Rubisco subunit gene expression were severely repressed by treatment with silver nitrate which could mean that the treatment, causing the production of ROS, induced senescence (Navabpour et al. 2003). However, the group did not observe expression of the senescence-specific *SAG12* gene and found that a number of genes observed actually required other signalling pathways, such as the salicylic acid or jasmonic acid pathways, to be expressed (Navabpour et al. 2003). This implies that the role of ROS in regulating senescence may act by stimulating stress response pathways. Furthermore, the accumulation of ROS during senescence may be caused as a result of the degradation of the chloroplastic macromolecules (Buchanan-Wollaston et al. 2003) which could result in disequilibria between the capture and dissipation of energy (Wingler et al. 2004). If this is the case, ROS production must occur after senescence has been initiated and it is thus a product of the senescence process not an initiator.

The progression and order in which the chloroplast proteins are degraded is not exclusively governed by the availability of proteases as the susceptibility of the target enzyme is also a controlling factor (Hörtensteiner and Feller, 2002). Rubisco, for example, is protected from the action of proteases and trypsin by 2'-carboxy-D-arabinitol 1-phosphate (Khan et al. 1999) and glutamine synthetase is protected by methionine sulphoximine which binds to the glutamate receptor site (Thoenen and Feller, 1998).

During the breakdown of the thylakoid-bound proteins many structural changes occur: The number of plastoglobuli increases (possibly accepting the end products of chlorophyll catabolism, as previously suggested), the membranes disrupt and then finally disappear and the space between grana stacks increases (Hörtensteiner and Feller, 2002). Light harvesting complex II (LHCPII) is a major source of nitrogen for remobilisation, but its stability is dependent on its status as a pigment-protein complex with chlorophyll (Buchanan-Wollaston et al. 2003). Consequently, its disassembly first requires the detoxification of chlorophyll as this results in the exposure of the protein to the action of proteases (Hörtensteiner and Feller, 2002; Buchanan-Wollaston et al. 2003) (see Figure 1.1).

The disruption of the cellular membranes, including the thylakoid membranes, during senescence is caused by the peroxidation of lipids. An increase in the action of enzymes such as phospholipase D and lipoxygenase and the increase in expression of genes involved in transcribing these enzymes are features of senescence (Buchanan-Wollaston et al. 2003). Senescence is an energy-dependent process and enzymes of fatty acid β-oxidation are therefore very important as the acetyl CoA produced from the lipids can be used directly for respiration (Buchanan-Wollaston et al. 2003). Earlier studies for dark-induced senescence initially suggested that fatty acids can also be converted to sugars by gluconeogenesis. However, no evidence for the expression of the glyoxylate cycle genes for malate synthase and isocitrate lyase, which are required for gluconeogenesis, has been found in naturally senescing Arabidopsis leaves (Buchanan-Wollaston et al. 2005).

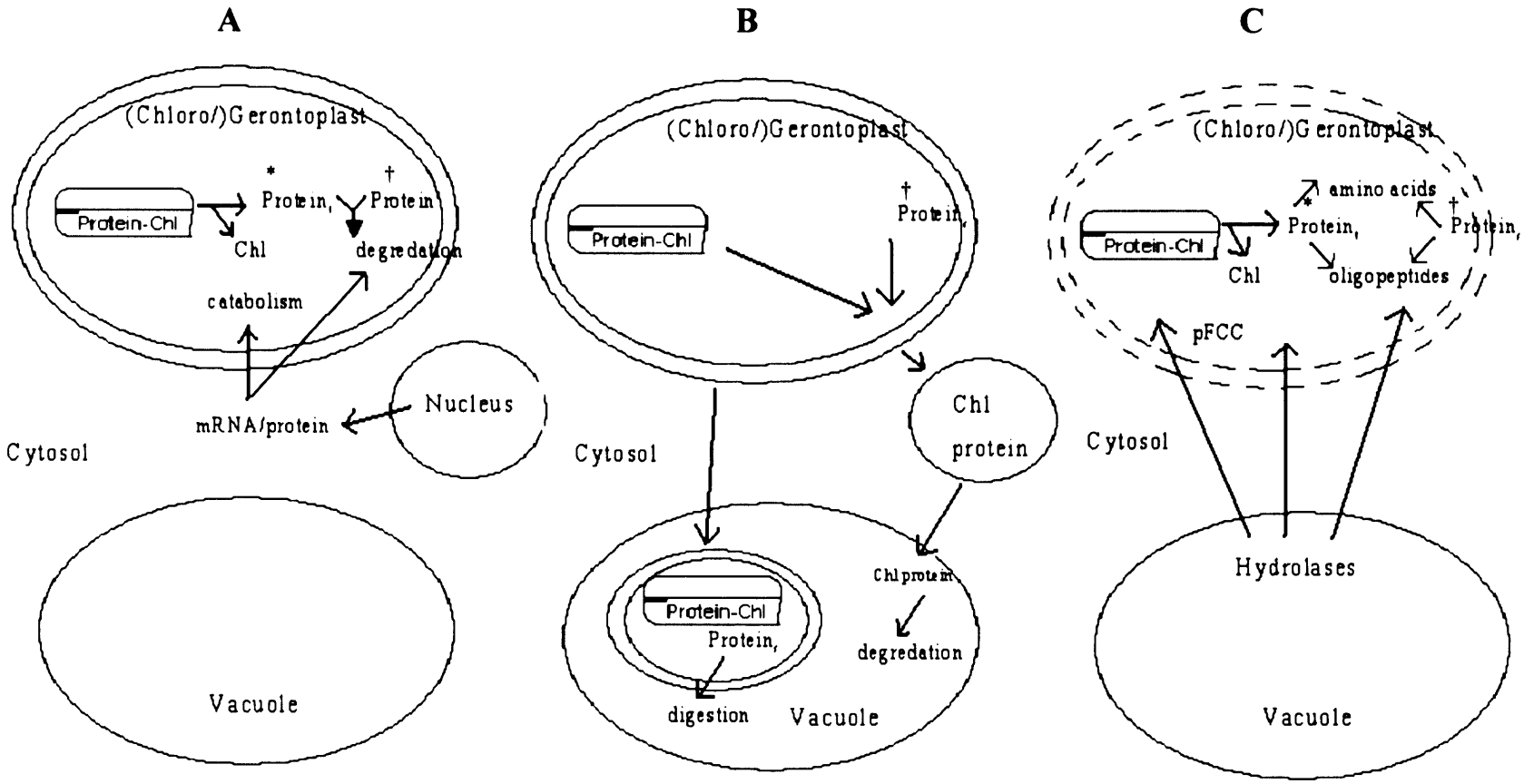


Figure 1.1 Degradation of the chloroplast constituents (by Hörtensteiner and Feller, 2002).

A: Enzymes encoded in the nucleus degrade proteins and chlorophyll within the chloroplast. B: Proposed method of the catabolism of chloroplast constituents in the vacuole of a) entire chloroplasts or b) chloroplast vesicles. C: After the loss of membrane integrity chloroplast constituents are degraded by extraplastidial enzymes.

*Protein: thylakoid-bound proteins. †Protein: stromal proteins.

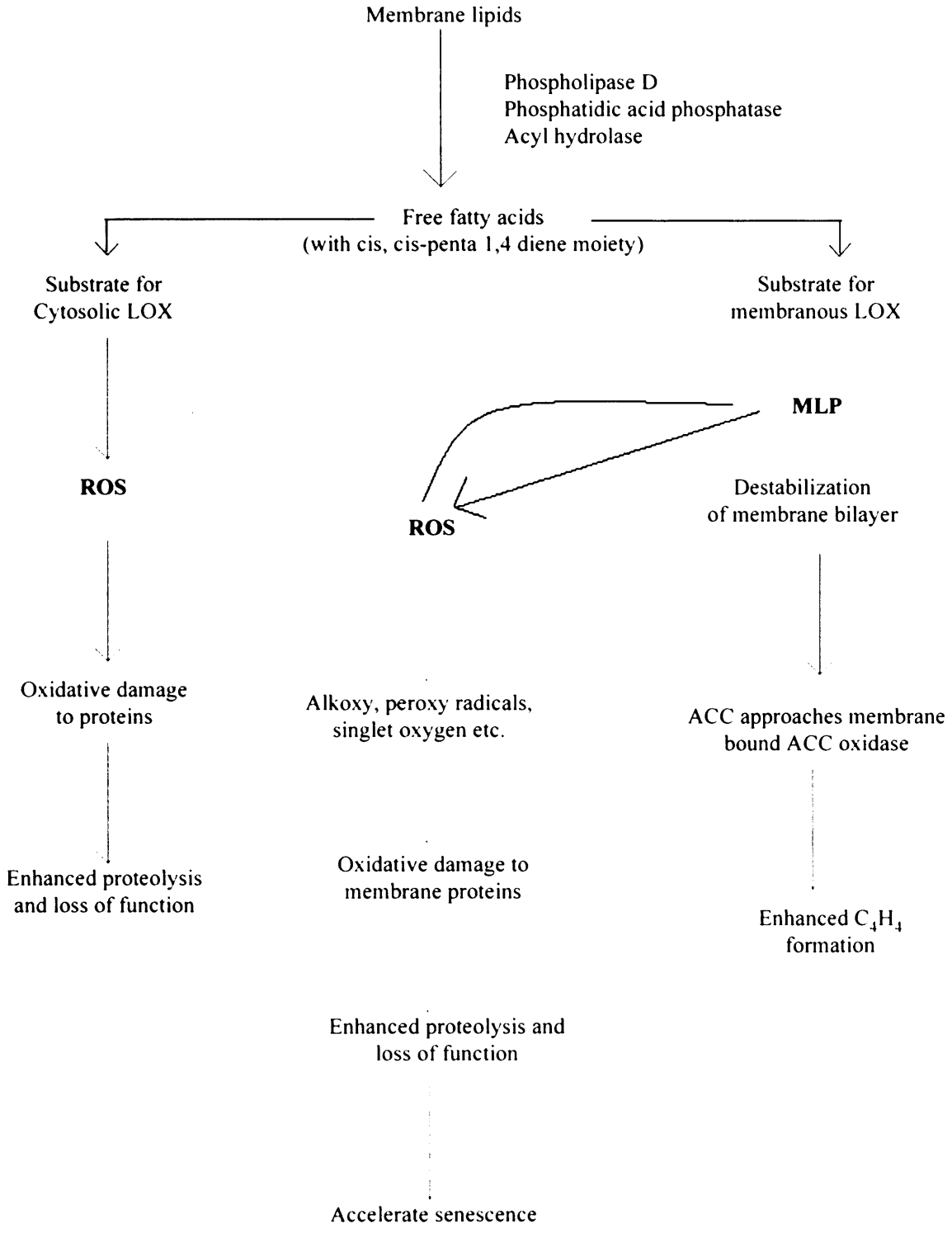


Figure 1.2 Hypothetical model of lipid damage by reactive oxygen species during senescence (Bhattacharjee, 2005).

As previously mentioned, the role of reactive oxygen species (ROS) in the degradation of the stromal enzymes has so far proved elusive, but ROS have been demonstrated to be active in lipid peroxidation during senescence (Figure 1.2). Lipid peroxidation is a source of ROS such as singlet oxygen and peroxy radicals through the action of lipoxygenase (Bhattacharjee, 2005). For the production of ROS an available source of Fe is required, which becomes increasingly available as senescence progresses. The explanation for this is unclear but possible explanations are changes in decompartmentalisation or the degradation of metalloproteins which are thought to increase the level of catalytic Fe and are a feature of senescence (Bhattacharjee, 2005).

1.1.4. Nutrient recycling

In addition to nitrogen, other essential nutrients are recycled during senescence (Table 1.1). There are species-specific differences in the remobilisation of elemental nutrients. For example, in Arabidopsis and pea no recycling of Mg occurs whereas in wheat and soybean it is recycled (Himelblau and Amasino, 2001.) There is also a difference between recycling during nutrient deficiency and during developmental senescence. Cu, Fe, Mo or Zn deficiency is most acutely observed in the young leaves, rather than the old leaves, of plants deprived of these nutrients. The explanation for this is that these nutrients are poorly transported in the phloem but as Himelblau and Amasino (2001) demonstrated, these nutrients appear to be effectively mobilized during developmental senescence, indicating that nutrient stress disrupts mobilization. The method of operation and the components of the nutrient remobilisation pathway remain largely unknown. The method of transport is almost

Element	% decrease between DAG* 27-37
Nitrogen (N)	85.4
Carbon (C)	37
Phosphorus (Ph)	78.4
Potassium (K)	85.3
Sulphur (S)	66.6
Chromium (Cr)	69.8
Copper (Cu)	56.9
Iron (Fe)	57.4
Molybdenum (Mo)	78.8
Zinc (Zn)	56
Sodium (Na)	No Change
Magnesium (Mg)	No Change
Calcium (Ca)	No Change
Nickel (Ni)	No Change
Manganese (Mn)	No Change
Cobalt (Co)	No Change

*DAG: Days after Germinating

Table 1.1. Percentage decrease in nutrients stored in Arabidopsis leaves between 27-37 DAG (from Himelblau and Amasino, 2001).

certainly via the phloem and consequently phloem accessibility and the mobility of the various nutrients reflect the efficiency with which they are mobilized (Bukovac and Wittwer, 1957). The identity of carrier proteins to mobilize free nutrients from the degraded chloroplast to the young leaves or seeds remains to be elucidated, but these proteins probably have to carry out a number of functions prior to transport including chemical modifications, intracellular trafficking and sequestration. Two genes were proposed by Himelblau and Amasino (2001) based on their identification as copper transporters in yeast. The first, an intracellular copper chaperone, *CCH* (Himelblau et al. 1998) had been shown to be expressed at the mRNA and protein level during senescence and was active in the phloem, which was highly suggestive for a role in long distance copper transport (Mira et al. 2001). The second, *RAN1* (*RESPONSIVE-TO-ANTAGONIST1*) was found to encode a copper-transporting ATPase (Hirayama et al. 1999) that is also expressed during senescence (Himelblau and Amasino, 2001). The authors of this study identified plants with T-DNA inserts within these genes and observed their growth and copper transport compared to the wild type plants. Unfortunately, no difference between the wild-type and the *cch-1* mutant was observed and *ran1-4* failed to develop normally (Himelblau and Amasino, 2001). The conclusion of this work was that *CCH* may comprise a redundant gene and that *RAN1* is involved in cellular maintenance and housekeeping during development and is not senescence specific. As the mutation in the latter gene compromised the plant health, no conclusion about a role in senescence could be drawn (Himelblau and Amasino, 2001). This latter study emphasizes the difficulty in identifying steps in the nutrient re-mobilizing pathway.

In the transport of nitrogen the modifications that occur prior to re-mobilization are poorly known and very few enzymes that metabolise amino acids during senescence have been identified (Diaz et al. 2005). Two good candidates for this function, however, are glutamate dehydrogenase and threonine deaminase (Masclaux-Daubresse et al. 2005). Changes in the proportions of glutamate (Gln) and asparagine (Asn) have been observed during senescence which is unsurprising as these are the two major amino acids transported in the phloem out of the senescing leaves (Diaz et al. 2005). Interestingly, the content of these two amino acids decreases to a greater extent in late senescing lines of Arabidopsis, implying that they were more effectively interconverted or remobilised from plants that exhibit greater longevity (Diaz et al. 2005). Glutamate (Glu) and glutamine (Gln) are major amino acids produced by the assimilation of mineral nitrogen. Aspartate (Asp) is produced from the transamination of glutamate (Glu) and, itself, is involved in the biosynthesis of several other amino acids including isoleucine (Ile) and leucine (Leu). As senescence progresses the levels of Asp were observed to decrease but the levels of Ile and Leu increased suggesting that the synthesis of these two amino acids depleted the available Asp (Diaz et al. 2005). The levels of γ-aminobutyric acid (GABA) also increases with age, similar to Ile and Leu. The explanation for this is unclear but possible functions include amplifying stress signals through the enhancement of ethylene synthesis, acting as a transient nitrogen storage compound or as an anaplerotic compound during stress-related metabolism (Diaz et al. 2005; Kinnersley and Turano, 2000). A correlation between proteolysis and the expression of two nitrogen assimilation enzymes, glutamate dehydrogenase (GDH) and cytosolic glutamine synthetase (GS1) has been observed in tobacco depending on leaf age (Masclaux et al. 2000). The expression of chloroplastic glutamine synthetase (GS2) and nitrate

reductase (NR) was found to decrease in an age-dependent manner, whereas the cytosolic glutamine synthetase (GS1) and GDH expression was found to be more persistent. These two enzymes show opposite reactions to sugars in that GDH is decreased by sugars, whereas GS1 is induced by glucose and sucrose (Masclaux-Daubresse et al. 2005). Both enzymes were stimulated by amino acids. Leaf sugar contents of tobacco plants increase with age and, consequently, the contents of GDH and GS1 reflect this in that an increase in GDH aminating activity was observed in young source leaves where sugar contents were lower and content of NH_4^+ was higher (Masclaux et al. 2000). The relative contents of GS1 and GS2 were found to alter with ageing. A progressive decrease in the content of GS2 and also a second chloroplastic nitrogen assimilation enzyme, ferredoxin-dependent glutamate synthase (Fd-GOGAT), occurred in parallel with an increase in GS1 during leaf ageing (Masclaux et al. 2000). This suggests that GS2 and Fd-GOGAT are responsible for ammonia re-assimilation and glutamate recycling in intact chloroplasts but that GS1 is operational in the ageing leaf (Masclaux et al. 2000) to sustain the synthesis of glutamine (Masclaux-Daubresse et al. 2005) for phloem export out of the senescing leaf.

The difference between GS1 and GDH expression in response to sugars reflects their different roles in senescence. Sugars have been observed to induce the expression of early senescence associated genes (SAGs) and represses late SAGs (Paul and Pellny, 2003). The expression patterns of GS1 and GDH, therefore, are reflective of an early and a late SAG, respectively. This indicates that GS1, the early SAG, may be involved with the synthesis of glutamate and GDH, the late SAG, may be involved in catabolic processes (Masclaux-Daubresse et al. 2005).

1.1.5. Genetic control of senescence

Over recent years a number of studies, employing the latest genetic technology have identified large numbers of genes upregulated during senescence (e.g. Buchanan-Wollaston et al. 2003; Gepstein et al. 2003; Lin and Wu, 2004; Pourtau et al. 2006; Masclaux-Daubresse et al. 2007). Genes specific to senescence are known as senescence associated genes (SAGs) and are involved in encoding a wide variety of enzymes, including proteases, chlorophyllase, carbon and nitrogen metabolising enzymes and post transcriptional regulators (Lin and Wu, 2004). Genes involved in stress responses such as cold (Masclaux-Daubresse et al. 2007; Appendix 4.3), wounding (Buchanan-Wollaston et al. 2005) and drought (Gepstein et al. 2003) have also been observed to be expressed during senescence. Other genes are down-regulated during senescence. Specifically, genes involved in photosynthesis and storage tend to be repressed and genes involved in remobilisation and catabolism are up-regulated.

The degradation of the nitrogen-containing chloroplast proteins is essential in the recovery of nutrients. It is unsurprising, therefore, that genes encoding protease enzymes are expressed during senescence. Genes involved in the ubiquitination cascade are also expressed during senescence (Gepstein et al. 2003). Furthermore, a mutation in the *ORE9* F-box protein, which is involved in the ubiquitination and subsequent proteolysis of target proteins through an interaction with the SCF complex, was found to delay senescence (Woo et al. 2001). This could imply that

ORE9 may act as an instigator of senescence by acting to degrade a key repressor of senescence via the ubiquitin pathway (Gepstein et al. 2003).

Cysteine proteases such as a cathepsin protease (Gepstein et al. 2003) and the papain-like cysteine protease, encoded by *SAG12* (Otegui et al. 2005) are upregulated. Interestingly, despite the majority of cellular proteins being located in the chloroplast, the location of a number of proteases is within the central vacuole, including the transcripts of *SAG12* (Otegui et al. 2005). The understanding of the role of the central vacuole in the degradation of cellular components is currently rudimentary but the discovery that, in addition to the large vacuole, other smaller peripheral vacuoles exist in tobacco indicates that these may be the location of proteolysis (Otegui et al. 2005). The mechanism by which these smaller vacuoles appear is also not clear but a possible candidate is autophagy. This is the process in which components of the cytosol and organelles are transported to the vacuoles in specialized vesicles to be degraded (Gepstein et al. 2003). This process has been better established in yeast, but the identification that orthologs of the *APG* genes (*AUTOPHAGIC GENES*) of yeast have been identified in Arabidopsis supports this theory (Otegui et al. 2005). The analysis of Arabidopsis autophagy gene (ATG) knockout mutant such as *At*ATG*18a* revealed that these mutants show an accelerated senescence phenotype and show a hypersensitive response to nutrient limitation (Xiong et al. 2005). This indicates that *ATG* gene expression does not act as an instigator of senescence, but has an important role in the re-mobilization of nutrients (van der Graaff et al. 2006). The increase in expression of ATG genes coincides with the first visible signs of chlorophyll degradation. It has been previously suggested that the ATG system is not senescence specific and the observation that some members of this family are expressed early in

plant development (4 weeks) supports this (van der Graaff et al. 2006). However the very large increase in the number of ATG genes expressed when senescence becomes visible (19 out of 21) observed by van der Graaff et al. (2006) rather suggests that they may play an essential role in nutrient recycling during senescence.

Nucleic acids are degraded during senescence and genes involved in ureide metabolism have been implicated, concurrently they show increased expression during senescence along with several nuclease genes (Buchanan-Wollaston et al. 2003). During senescence, glutamine, the major amino acid exported in the phloem, is formed from glutamate, while glutamate is also decarboxylated to GABA. Consequently, genes for cytosolic glutamine synthetase and for glutamate decarboxylation are up-regulated during developmental senescence, together with two genes for glutamate receptors (Buchanan-Wollaston et al. 2005). This regulation of genes involved in nitrogen metabolism is also found in sugar-induced senescence (Pourtau et al. 2006).

Himelblau and Amasino (2001) reported that there was an about 80% reduction in leaf phosphorous during senescence. A major source of leaf phosphorous is within nucleic acids and so their disassembly also frees this essential nutrient. A proposed method of phosphorous transport is via AtPT2 which has been demonstrated to serve this function (Muchhal et al. 1996).

Unsurprisingly, considering that the principal function of senescence is recycling of nutrients, increased expression of genes involved in amino acid (Gepstein et al. 2003;

Pourtau et al. 2006), peptide (Buchanan-Wollaston et al. 2003) and sugar transport (Buchanan-Wollaston et al. 2005; Pourtau et al. 2006) has been observed.

Lipid peroxidation is clearly an important senescence process as plants showing inhibited membrane lipase expression display delayed senescence. An example of this is the *SAG101* gene which encodes an acetyl hydrolase, the product of which releases oleic acid from triolin (Yoshida, 2003). The inhibition of this gene by antisense suppression retards senescence whereas its overexpression results in acceleration. This implies that lipid peroxidation could induce senescence (Yoshida, 2003). Two possible mechanisms by which this could happen are through the production of ROS (as previously discussed) or through the release of carbohydrates resulting in sugar signalling. The role of sugars in regulating lipid catabolism has been highlighted in senescence studies in which dark incubation has been used to induce senescence. Dark incubation results in a rapid decrease in sugars due to the inhibition of photosynthesis and is paralleled by an increase in the expression of lipid catabolism, particularly β-oxidation activity in plants undergoing dark-induced senescence compared to developmental senescence (Graham and Eastmond, 2002; Buchanan-Wollaston et al. 2005). As discussed above, the increase in β-oxidation activity is not complemented by an increase in genes involved in the glyoxylate cycle which are required for gluconeogenesis. This implies that the acetyl CoA released from the β-oxidation of lipids is respired directly (Buchanan-Wollaston et al. 2005).

Two lipoxygenase genes, *AtLOX1* and *AtLOX2* and a fatty acid α-dioxygenase gene, *AtαDOX1* are involved in the biosynthesis of jasmonic acid and have been observed

to be strongly up-regulated during senescence (van der Graaff et al. 2006). In developmental senescence *AtLOX2*, an allene oxide synthetase gene *AtAOS1*, and 12-oxophytodienaote reductase, *AtOPR3*, are strongly regulated. In dark-induced senescence *AtLOX1* and *AtαDOX1* are strongly induced in detached leaves only, implying that these two genes play a role in a stress response to wounding (van der Graaff et al. 2006). Recently, it has been established that *AtαDOX1* is involved in the production of oxylipin which protects plants from oxidative stress and cell death (De Leon et al. 2002) defining its role as a stress responsive gene. No induction of genes involved in jasmonate biosynthesis was observed in the aforementioned study in dark incubated whole plants implying that jasmonic acid does not play a major regulatory role in dark-induced senescence (van der Graaff et al. 2006).

The expression of *AtαDOX1*, in addition to jasmonic acid, also requires ethylene for expression (Buchanan-Wollaston et al. 2005). A second jasmonate and ethylene dependent gene that is transiently up-regulated during dark induced senescence is *AtERF1* (Lorenzo et al. 2003). This gene is activated when *AtEIN3*, a downstream component of the ethylene signalling pathway, binds to its response element. *AtEIN3* expression is significantly up-regulated in natural and dark induced senescence (van der Graaff et al. 2006).

The use of dark-induced senescence of detached or individual leaves to observe gene expression has been employed in many studies (Gepstein et al. 2003; Lin and Wu, 2004; van der Graaff et al. 2006) and certainly this method is effective in inducing yellowing, chlorophyll loss and expression of some SAGs similar to developmental senescence (Weaver and Amasino, 2001). Overlap between the expression patterns of

dark-attached or detached leaves and natural senescence has been observed and offers an insight into which SAGs are responsive to stress conditions and which are ageing induced (van der Graaff et al. 2006). In a transcriptome study, the aforementioned authors observed the expression of 3,513 genes in plants that underwent natural senescence (NS) and 1,833 and 2,158 genes expressed during incubation of attached, individually darkened leaves (DIS) and incubation of detached leaves (DET), respectively (van der Graaff et al. 2006). Many of the genes observed in NS increased in expression progressively and so it is probable that some of the genes whose expression was absent in the dark treatments, simply did not have enough time for their expression levels to be detected. Interestingly, the comparison between the proportion of genes that were up-regulated or down-regulated depended on treatment. NS was the only treatment in which more genes were up-regulated rather than down-regulated (van der Graaff et al. 2006). The authors observed that transporter activity was increased in 21 out of 80 transport families in all senescence treatments. The largest increases were observed in NS but all treatments showed upregulation of families such as a cationic amino acid transporter and a K^+ uptake permease. This is consistent with the mass degradation and remobilisation that occurs during senescence (van der Graaff et al. 2006). Plastidial transporters tended to be down regulated compared to those located on the plasma membrane of which expression was increased in both NS and DET. The explanation suggested by van der Graaff et al. (2006) is that this reflects the reduction in the export of photoassimilates during senescence. It is possible, however that the few (14) plastidial transporters that were up-regulated have a direct role in recycling nutrients from the degrading plastid (van der Graaff et al. 2006).

Protein kinases are up-regulated, which is evidential that kinase-signalling cascades are functional during senescence (Buchanan-Wollaston et al. 2005). Many of these protein kinases belong to a family of receptor-like kinases (RLKs) which share kinase domain families with some of those found in mammals and insects. Of the RLKs that are up-regulated during senescence the majority were only found in NS and not in DIS or DET (van der Graaff et al. 2006). The role that RLKs play in senescence remains unclear, and possibly non-existent, as it remains to be established whether RLKs are plant specific (Gutierrez et al. 2004).

Translation regulator genes are also upregulated during senescence including DNA and RNA binding proteins (Buchanan-Wollaston et al. 2005). Conversely to RLKs, the expression of many transcription factors appears to be specific to plants (van der Graaff et al. 2006). In the comparison of 1,985 putative transcription factors only 1.6% were specific to taxa other than plants whereas 32% were plant specific, of which 21% were differentially regulated during senescence (van der Graaff et al. 2006). This implies that transcription factors are major components both in the induction and regulation of leaf senescence (van der Graaff et al. 2006).

From the examples presented it is clear that the regulation of senescence involves multiple pathways and is highly sensitive and responsive to changing environmental stimuli.

1.1.6 Is senescence a form of programmed cell death?

The definitions of both senescence and programmed cell death (PCD) are varied, overlapping or identical depending on the comprehension of the author to the meaning of either. For example senescence has been defined as: "The deteriorative processes that are the natural causes of death" (Medawar, 1957). Programmed cell death has been described as: "A specific series of events that culminate in cell death" (Laytragoon, 1998). PCD was a phrase originally coined in the 1960's in reference to animal sciences and it was not introduced into general use in plant sciences until the 1980's (Lascaris and Deacon, 1991). Senescence is derived from the Latin, *senescere*, meaning to age (van Doorn and Woltering, 2004). A difference between the accepted notion of senescence and PCD is spelled out in the name of the latter. Senescence is a term used in reference not just to individual cells but tissues, organs or whole organisms whereas programmed cell death, by very definition, is specific to the activity of a cell or cells and therefore its use to refer to organs, tissues or individuals is, at best, imprecise (van Doorn and Woltering, 2004). One long-standing argument for a process of senescence in its own right is that certain senescence processes have been observed to be reversible, i.e. do not result in cell death. One such process is de-greening which has been observed to occur in a number of plant organs including leaves, sepals and fruit peel. The sepals of *Helleborus niger*, for example, lose chlorophyll in order to attract pollinators and then re-green once pollination has taken place (Salopek-Sondi et al. 2002). It could be argued that this is not a process of senescence as, by the general definition, it is not a process leading up to death. Nevertheless, the process involved must be originating from senescence. Adaptation of senescent leaves in the evolutionary development of

flower petals has been implicated previously (Thomas et al. 2001) and, if correct, demonstrates that senescence-like-processes are dynamic and adaptable traits that do not inevitably result in death.

The definition of "death" has also been called in to question because, as Thomas et al. (2003) explain, it is difficult to establish which of the chemical and structural changes occur pre- or post-mortem. Processes such as free radical cascades, macromolecule cleavage and oxidation are all characteristic of senescence but also occur in the early stages of necrotrophic and necrochemical attack following death (Thomas et al. 2003). Furthermore, the notion of genetically programmed death should be approached with caution as it is not necessarily the cell being programmed to die but the programming of internal proteases (for example) that leads to destruction and eventually death (Thomas et al. 2003).

As the term PCD originated in animal research and its role in this field of science is much less controversial, comparison between the processes occurring in plants and animals could potentially clarify the conundrum. In animal studies the initiation of PCD appears to depend on the re-activation of pro-apoptosis genes (Thomas et al. 2003), which results in an increase in caspase activity and DNA laddering then ensues. The closest functional homologues to caspase in plants are cysteine proteases and the activity of these enzymes is certainly up-regulated during senescence (Buchanan-Wollaston, 1997). The use of DNA laddering as evidence for PCD is still inconclusive as nucleic acids are a ready source of phosphorous and will thus be the target of the action of phosphatases and so some laddering will occur without the need for a specific programmed function (Thomas et al. 2003).

Senescence is nearly always defined as being a developmental process (Buchanan-Wollaston et al. 2003; Pourtau et al. 2004; Wingler et al. 2005) whereas the terminology of PCD implies that the function of the process is to cause death. In terms of the function of senescence the purpose is to reclaim stored nutrients for use elsewhere to maximise the chances of survival. The *death* of the cell or the tissue is incidental to this process. PCD as a term does not, therefore, reflect nor do justice to the processes involved. "Programmed cell recycling" or "programmed nutrient re-distribution" would more accurately describe the processes at hand. Therefore, PCD is a relevant term when, specifically, the death of the cell is the beneficial function such as destruction of a cell to prevent the spread of a pathogen.

1.2 Sugars and senescence

1.2.1 Does sugar starvation induce senescence?

The breakdown of the chloroplast and the loss of photosynthetic function could, logically, result in a decline in leaf carbohydrates and through starvation result in senescence.

Senescence can be induced in detached leaves placed in darkness or in individually shaded attached leaves. These methods have been employed in a number of studies to investigate senescence mechanisms (Thimann and Satler, 1971; Biswal and Biswal, 1984; Lin and Wu, 2004). Interestingly, however, senescence is not induced if whole

plants are placed in darkness; that is to say chlorophyll a/b-binding (CAB) proteins are not degraded, visible yellowing does not occur and *SAG12* is not expressed (Weaver and Amasino, 2001). However, in individually shaded leaves that were still attached to the plant, all these characteristics of senescence were observed (Weaver and Amasino, 2001). To test whether the response in individually shaded leaves could be reversed, the aforementioned authors removed the shading "mittens" from the attached leaves. Senescence was not relieved by this action and continued after removal of the constraint. Furthermore, the leaves of darkened whole plants were less senescent than plants that had been left to develop naturally without dark incubation (Weaver and Amasino, 2001).

To investigate at what level regulation of shading was taking place in individually darkened leaves, i.e. cellular or whole organ, Weaver and Amasino (2001) allowed light to penetrate to the shaded leaf through a small hole in the shading bag. The results showed that the localised area that had received light remained green, whereas the rest of the leaf yellowed, thus demonstrating that regulation is highly localised. The authors of this paper do not attempt to explain why detached and individually darkened leaves senescence whereas whole plants in darkness do not, but a possible candidate for controlling these responses is sugars.

Dark incubation inhibits photosynthesis and rapidly causes sugar starvation (van der Graaf et al. 2006). However, if sugar starvation were responsible for causing senescence the whole plant incubation treatment would be expected to produce the most senescent phenotype, which it does not (Weaver and Amasino, 2001). Indeed, it appears that the very reverse is being observed, that sugar starvation inhibits

senescence. An explanation as to why individually shaded leaves still senesced when they, too, must be sugar starved is that carbohydrates produced in other unshaded leaves could be transported to the photosynthetically compromised leaves. A number of recent studies have identified that application of sugars or the increase of leaf sugar contents by treatment with high light actually accelerates senescence (Wingler et al. 1998; Pourtau et al. 2004; Rolland and Sheen, 2005) and also that sugar contents progressively increase as senescence develops rather than depleting (Pourtau et al. 2004; Diaz et al. 2005), thus indicating that sugars may play an important role as signals or regulators of senescence. This hypothesis is discussed in greater detail in Section 1.2.2.

If sugar starvation as an instigator of senescence in leaves appears unlikely, quite the opposite can be said for the role of sugar starvation in cut flowers. Although, as in leaf senescence, no definitive role for either sugar or starvation induced senescence has been proven, sugar starvation appears a likely candidate. Structural, genetic and metabolic changes that occur in the senescing petal are very similar to those observed in sugar-starved organs (van Doorn, 2004). Structurally the multiple small vacuoles fuse to produce one larger structure in a manner reminiscent of sugar-starved plants (van Doorn, 2004). Metabolically, the senescence of flower petals involves proteolysis and degradation of lipids and the main amino acids to be transported in to the phloem are asparagine (Asn) and glutamine (Gln). Similarly, sugar-staved maize root tips also utilize Asn and Gln for re-mobilization and undergo rapid proteolysis and lipid degradation. Genetically, the up-regulation of genes involved in regulating these processes was observed (proteases, lipases etc) (Brouquisse et al. 1998). An argument against sugar starvation inducing senescence in cut flowers is that flower

petals often contain large amounts of carbohydrate. On the other hand, the application of exogenous sugars can delay senescence. With regards to the quantity of carbohydrate within flower petals, van Doorn (2004) argues that the various tissues of flower petals are often at different stages of development so the content of sugars may represent the content in healthy cells not those of the senescent cells. The apparently delaying effect of sugar application is also not conclusive as sugars may act to harmonise the osmotic status and water relations of the cut flower and also repress the accelerative effect of ethylene, rather than actually signalling specifically for senescence.

1.2.2. Sugar signalling

Sugars contribute a fundamental part of plant life. They are the end products of photosynthesis and provide the building blocks for all plant compounds including amino acids. More recently sugars have been recognised to act as molecular signals that produce altered gene expression, similar to hormones. A definition of sugar signalling is:

"..the interaction between a sugar molecule and a sensor protein in such a way that a signal is generated. The signal then initiates signal transduction cascades that result in cellular responses such as altered gene expression and enzymatic activities." (Smeekens, 2000).

Sugar signalling affects a large range of developmental processes in plants (Gibson, 2005), including germination (Price et al. 2004), leaf development (Raines and Paul 2006), stress response (Ehness et al. 1997) and senescence (Pourtau et al. 2004; Diaz

et al. 2005; Wingler et al. 2006; Masclaux-Daubresse et al. 2007). The way in which sugar signalling occurs has not been entirely elucidated, but it has been shown that multiple sensors and pathways exist (Sheen et al. 1999; Rolland et al. 2002; Halford and Paul, 2003; Rolland et al. 2006). One well established sugar sensor is hexokinase, a sugar phosphorylating enzyme (Jang and Sheen, 1997) which was first characterised in yeast (*Sacharomyces cerevisiae*). Hexokinase (HXK) is a dimeric cytosolic enyzyme which functions in glycolysis (Sheen et al. 1998). Possible signals down stream of hexokinase are calcium dependent protein kinase (CDPK), protein phosphatase and transcription factors (Sheen et al. 1999). The study of yeast has identified stages of the sugar signal transduction pathway involved in the repression of photosynthesis that may be conserved in plants. Two such genes are *GLC7* and *REG1*, the former has been definitively identified to encode a protein phosphatase and the latter is assumed to do so (Jang and Sheen, 1997). Inhibitors of these two genes block the repression of photosynthesis-related genes that has been induced by sugars. One inhibitor, okadaic acid can mimic the effect of glucose and cause the subsequent repression, further supporting the role of protein phosphatases in regulating the characteristic sugar repression of photosynthesis (Jang and Sheen, 1997).

As a sugar sensor, HXK has been observed to play an essential role in the sugar-regulated repression of photosynthesis genes such as Rubisco (Smeekens, 2000). When cells of *Chenopodium rubrum* were cultured in the presence of glucose, Rubisco transcript levels were repressed, but non-phosphorylatable glucose analogues such as 6-deoxyglucose (6-dGlc) or 3-O-methylglucose (3-O-mGlc), had no effect (Smeekens et al. 2000). A recent micro-array study was carried out to investigate the expression of genes in response to glucose, 3-O-methylglucose and 6-deoxyglucose.

Although 200 genes were shown to be responsive to glucose, none were differentially regulated by either glucose analogue (Villasden and Smith, 2004).

The use of HXK mutants, such as the *gin2* mutants of Arabidopsis, has further established the role of this enzyme in mediating sugar signalling pathways. When grown under low light intensities, thus limiting endogenous glucose levels, *gin2* mutants and wild type plants were phenotypically similar. When high light was applied, however, the phenotype became markedly different with the wild type showing accelerated development whereas the mutant remained small with dark green leaves (Moore et al. 2003). Similarly a parallel between reduced leaf glucose contents and the retaining of a high F_v/F_m, compared to wild-type L*er* plants, was observed in the Arabidopsis mutant, *gin2-1*, indicating altered sugar metabolism, in addition to reduced glucose sensitivity as a result of the hexokinase mutation (Pourtau et al. 2004). However, studies of *HXK1* mutants have also demonstrated that signalling can occur independently of HXK as these plants still display evidence of signalling through gene expression, inflorescence development, leaf expansion and senescence (Moore et al. 2003). Furthermore the *gin2-1* mutant plants senesced more quickly on low nitrogen medium plus 2% glucose rather than without glucose, suggesting a role for hexokinase-independent signalling, in addition to the role of HXK in developmental processes (Pourtau et al. 2004).

Another important sugar signalling pathway in plants involves a plant protein kinase that is related to the Sucrose Non-Fermenting 1 (SNF1) protein kinase of yeast (for review see Halford and Paul, 2003; Halford et al. 2003; Halford et al. 2004). Due to its similarity with the yeast protein, the plant protein kinase is called SnRK1 (SNF1-

Related Protein Kinase 1). In yeast, SNF1 is activated by low glucose concentration and it is required for the activation of genes that are usually repressed by glucose, such as the *SUC2* gene for invertase. Due to the lack of invertase, the yeast *snf1* mutant can therefore not metabolise sucrose, hence its name. In plants, *SnRK1* genes are present in families, with, for example, three family members in Arabidopsis and between ten and twenty in barley (Halford et al. 2004). SnRK1 can be regulated at the transcriptional and post-transcriptional levels. Post-transcriptional regulation involves phosphorylation by an upstream kinase. Overall, SnRK1 appears to be activated by high sucrose or low glucose concentrations. SnRK1 itself acts by directly phosphorylating and thereby inactivating enzymes, such as nitrate reductase and sucrose phosphate synthase, but also by regulating gene expression, e.g. of sucrose synthase genes (Halford et al. 2003).

The role of sucrose as a signalling molecule has been demonstrated by determination of sucrose transporter activity in plants treated with sucrose. The transport of sucrose dropped 35-50% in plants treated with 100 mM sucrose compared to control plants but the transport of glucose and alanine remained unaffected (Chiou and Bush, 1998). Expression of the sucrose transporter gene is also repressed by sucrose, suggesting that export of sucrose out of the leaf by phloem loading can be down-regulated if the sink demand is low. In addition a role of sucrose transporters as sugar sensors has been suggested by Lalonde et al. (1999). The fact that sucrose is easily hydrolysed to glucose and fructose has made determining a specific signalling function difficult and the identification of many of the components of the signalling pathways remains to be elucidated. A model of the sugar signalling pathways is represented in Figure 1.3.

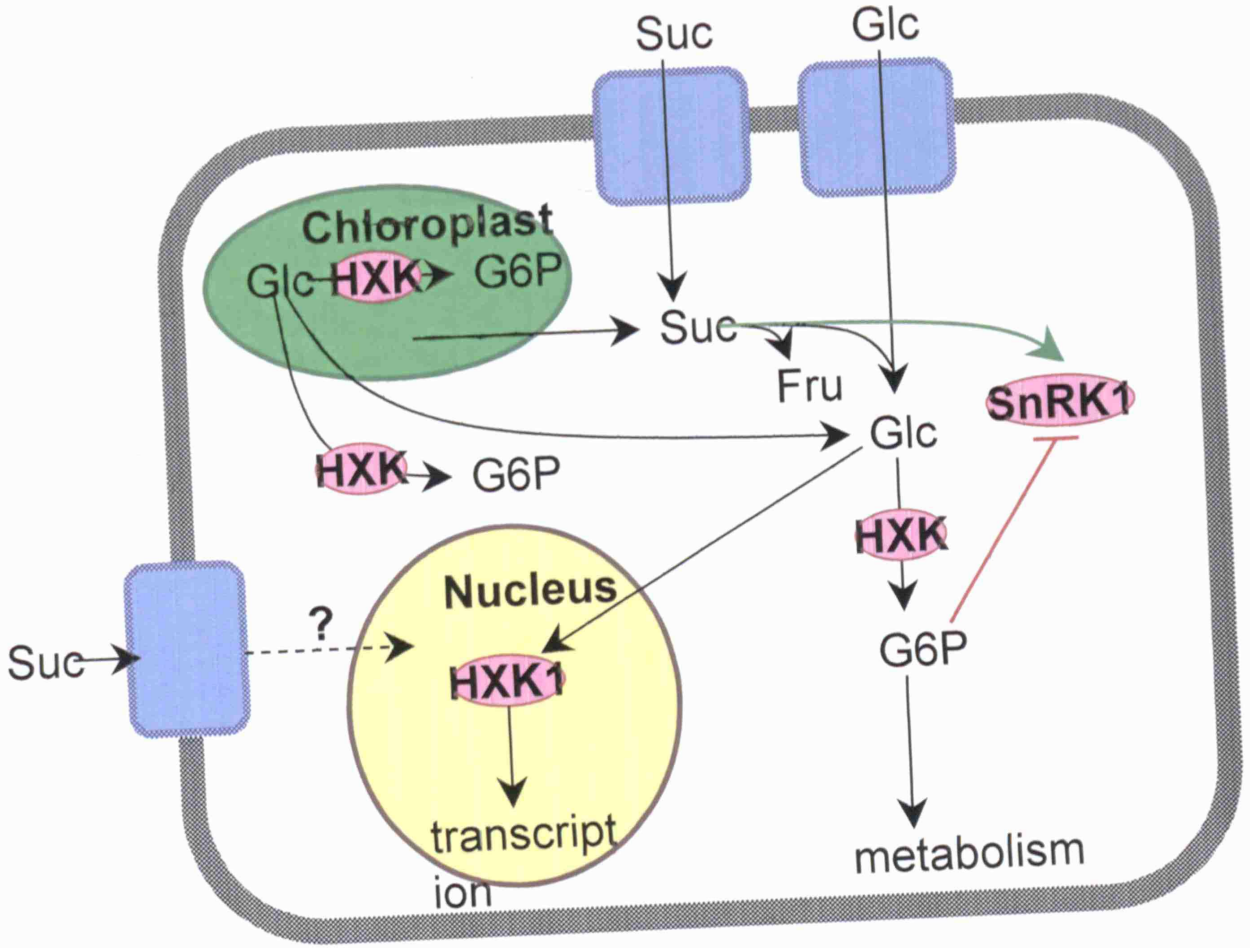


Figure 1.3 Model of sugar-sensing mechanisms in plants (modified from Rolland et al. 2006).
Sugar transporters are in blue. The green line indicates activation of SnRK1 by sucrose, the red line inhibition by glucose-6-phosphate. The dashed line indicates the possible involvement of sucrose transporter homologues in sugar signalling. Hexokinase 1 is found in the nucleus where it probably controls transcription.
Glc = glucose, G6P = glucose-6-phosphate, Fru = fructose, HXK = hexokinase, SnRK = SNF1-related protein kinase 1.

1.2.3 Sugar signalling during senescence

Leaf senescence is a plastic trait, that is to say it is regulated and altered according to environmental cues. Darkness can induce senescence as has been demonstrated in a number of studies (Gepstein et al. 2003; Lin and Wu, 2004; Buchanan-Wollaston et al. 2005). However, dark incubation of whole Arabidopsis plants does not induce senescence, indicating that senescence is not starvation induced (Weaver and Amasino, 2001). Instead, light seems to play a role in the regulation of senescence of individually shaded leaves, probably by simulating the effect of shading by other plants or leaves. Nevertheless, shading experiments point to a role of sugar signalling in the regulation of senescence (Wingler et al. 2005). For example, the shading of young leaves of sunflower and bean resulted in a decrease in sugars in the old, unshaded leaves, and delayed their senescence, suggesting that senescence is sugar inducible (Ono et al. 2001). Leaf senescence is accelerated when plants are grown in a combination of low nitrogen and 2% glucose (Pourtau et al. 2004; Wingler et al. 2004; Pourtau et al. 2006; Masclaux-Daubresse et al. 2007). Furthermore, the analysis of Affymetrix GeneChip data taken from plants grown in these conditions has demonstrated that glucose-induced senescence is characteristic of developmental senescence (Pourtau et al. 2006). The expression of the *SAG12*, for example, was greatly increased in the presence of glucose and this gene is recognised as senescence specific and is not inducible by stress (Weaver et al. 1998). This result was surprising since Noh and Amasino (1999) report that *SAG12* expression is down-regulated by treatment of detached Arabidopsis leaves with sugars. This discrepancy could indicate

that the effect of sugars depends on the developmental stage of the leaf, e.g. old leaves could be more responsive to sugar-induced senescence than young leaves. This is supported by a recent study by Araya et al. (2006) demonstrating that sugar treatment results in the down-regulation of photosynthesis in old source, but not in young sink leaves.

The hexokinase mutant *gin2-1* does not show the typical senescence-related hexose accumulation (Pourtau et al. 2006) and also displays a delayed senescence phenotype (Moore et al. 2003). When grown on medium with low nitrogen in combination with 2% glucose, senescence is delayed in *gin2-1* compared to the wild type, indicating a role of hexokinase in senescence signalling. Abscisic acid (ABA) can accelerate senescence and it is required for sugar signalling in young plants (Rook and Bevan, 2003), but abscisic acid signalling mutants (*abi* mutants) or abscisic acid synthesis mutants (*aba* mutants) still display accelerated senescence in response to glucose treatment demonstrating that ABA is not required for sugar-induced senescence (Pourtau et al. 2004). Opposite to the effect of ABA, cytokinins can delay senescence which is thought to occur through its interaction with sugar signalling (Wingler et al. 1998). The explanation for this is that cytokinin induces extracellular invertase thus increasing sugar utilization, decreasing sugar storage and delaying senescence (Balibrea Lara et al. 2004).

1.2.4 Interaction with nitrogen signalling

Nitrogen limitation can accelerate senescence but, interestingly, this appears to act through an interaction with sugars. Nitrogen deprivation results in an accumulation of

sugars (Paul and Driscoll, 1997). In addition, sugars were accumulated more strongly when Arabidopsis plants were grown at low nitrogen supply than at high nitrogen supply in the presence of 2% glucose (Pourtau et al. 2004). A possible explanation for this response is that high nitrogen supply leads to a greater demand for carbon skeletons for protein and amino acid synthesis (Winger et al. 2005).

Genetically both nitrogen and sugars affect gene expression although, perhaps surprisingly, glucose has a greater effect on the expression of nitrogen-related genes than nitrogen (Price et al. 2004). Nitrogen, however, appears to have the ability to override the carbon induced repression of photosynthesis related genes. For example, in conditions of high glucose (111 mM) combined with high nitrogen, normal growth and development was observed but when plants were grown with the same glucose treatment but at low nitrogen, senescence was accelerated and typical SAGs were expressed (Pourtau et al. 2004). Similarly, plants grown in elevated CO_2 conditions, which acts to increase leaf sugar contents, photosynthesis repression was not observed if nitrogen availability was kept high (Paul and Pellny, 2003). The relationship between sugar, nitrogen and CO_2 in regulating senescence is depicted in Figure 1.4 (Wingler et al. 2006).

In a study of tobacco the levels of glutamate dehydrogenase (GDH), glutamine synthetase (GS1), nitrate and ammonium were compared to carbohydrate contents (Masclaux et al. 2000). The authors observed that the youngest leaves contained very low levels of sugar, an increase was then observed in the middle, mature leaves and then a decrease in the oldest leaves. The nitrate content of the plants was low in young and mature leaves, but was higher in the old leaves. The ammonia content was

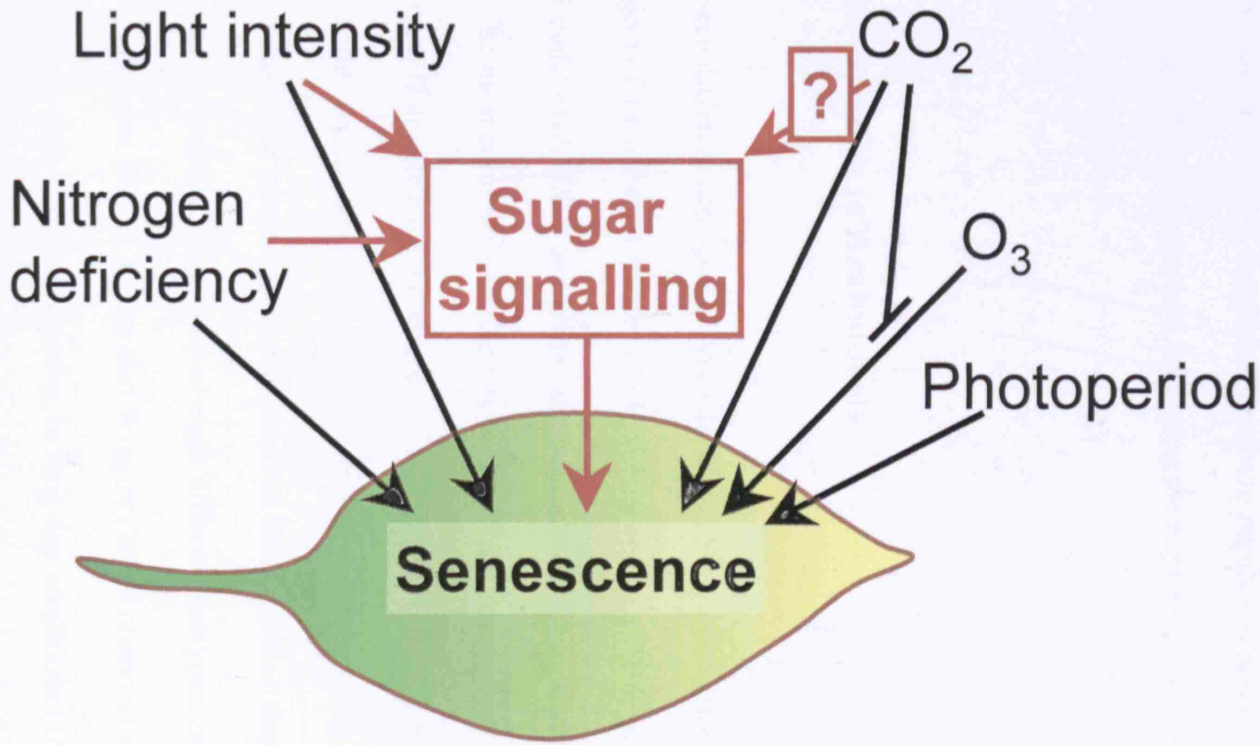


Figure 1.4 Hypothetical model of the interactions between sugars and nitrogen in regulating senescence.

Light intensity, nitrogen deficiency and elevated $C0_2$ result in sugar accumulation. Sugar accumulation acts as a signal for the initiation of senescence.

high in the young leaves then declined in the middle leaves. In the old leaves, ammonia content was high, similar to the young leaves (Masclaux et al. 2000). From this it can be observed that an inverse relationship exists between sugars and nitrogen (Masclaux et al. 2000). In particular, the carbon/nitrogen ratio was high in mature leaves, at the stage during which senescence may be first induced. The relationship between carbon and nitrogen is therefore thought to regulate senescence by acting as a signal for the activation of senescence, including the expression of senescence associated genes (SAGs).

1.3 Natural variation in Arabidopsis

Variety between different ecotypes of Arabidopsis with regards to flowering time has been the subject of a number of studies (Alonso-Blanco et al. 1998; El-Din El-Assal et al. 2001; Loudet et al. 2002) and characterisation of the different phenotypes is well recognised. The most commonly used ecotypes such as Columbia (Col) and Landsberg *erecta* (L*er*) are early flowering but a variety of other ecotypes are late flowering and often require a vernalisation period to induce flowering (Johnson et al. 2000). Natural variation in senescence has received little attention despite a wide range of phenotypes existing. In a study of eight different ecotypes, originating from geographically different origins, Levey and Wingler (2005) observed significant differences between ecotypes. Furthermore, in long day conditions (16 hours) the leaf senescence phenotype of the early senescing lines (Bay-0, Col-5, Nd-1 and L*er*-2) and late senescing lines (Kas-1-1 and Kas-1-2) were found to reflect the flowering phenotype (Levey and Wingler, 2005). The authors also observed an ecotype

dependent range of responses to day-length which they concluded could be the result of different levels of sugar accumulation or sensitivity (Levey and Wingler et al. 2005). The extent of variation between ecotypes is unsurprising as Arabidopsis is found in many different habitats including forests, swamps, agricultural fields and prairies (Luquez et al. 2006). The study of 45 ecotypes revealed bolting variation of 60-107 days and post-bolting longevity varied from 23-45 days (Luquez et al. 2006). These two studies reveal that Arabidopsis exhibits a wide range of senescence phenotypes which are reflective of its diverse genetic status and render it a good tool with which to dissect this complex process.

1.4 The Bay-0 x Shahdara recombinant inbred line population

The Bay-0 x Shahdara recombinant inbred lines (RILs) were first presented in 2002 (Loudet et al. 2002a). Formed from the cross between two distantly related ecotypes, Bay-0 from fallow land in Bayreuth, Germany and Shahdara from the Pamiro-Alay mountains of Tadjikistan the cross was designed to yield a population of genetically diverse individuals that could be used to probe complex traits. The population has since proved its worth as quantitative trait loci (QTL) analyses have been carried out on the population and loci involved in flowering variation (Loudet et al 2002a), nitrogen use efficiency (Loudet et al. 2002b), yellowing and anthocyanin accumulation (Diaz et al. 2006), carbohydrate content (Calenge et al. 2006) and water and anion interactions with nitrogen (Loudet et al. 2003) have been uncovered.

The population was produced from single seed decent (SSD); the parental ecotypes were crossed and the resulting progeny planted in individual pots and allowed to self fertilise. This process was repeated and the F7 seeds were used for experimentation (Loudet et al 2002a). The population was designed to be dedicated to quantitative trait loci (QTL) analysis a technique designed to dissect the genetic architecture of complex traits (Loudet et al. 2002a). Aside from QTL analysis, which was first used in the 1980's (Edwards et al. 1987), the study of traits has largely focused on the use of mutant plants. Although this method has proved its worth in innumerable studies the majority of traits are not governed by single genes but by multiple loci that may be uncovered by QTL analysis. The Bay-0 x Shahdara population has been extensively mapped using 38 microsatellite markers situated every 10-15 cM along the five chromosomes. The microsatellite motifs used were (AT)*n* or (AG)*n*, where $n>12$. The majority of QTL studies have used the Landsberg *erecta* (L*er*) x Columbia (Col) and L*er* / Cape Verde Island (Cvi) recombinant inbred populations. The Bay-0 x Shahdara population is greater in size and the use of a Central-Asian x European variety was predicted in their design to yield interesting phenotypic variation (Loudet et al. 2003). This prediction has proved fruitful as in the four years since the development of the RILs QTL involved in a wide variety of agronomically important traits have been mapped. One such example is nitrogen use efficiency. The study by Loudet et al. (2003) sought to identify QTL involved in nitrogen use efficiency in high and low nitrogen conditions. The genetic variation for this trait was calculated prior to the analyses and was found to be highly significant. Furthermore, approximately 50% of lines showed a transgressive phenotype compared to their parental accessions indicating that, as predicted, the accessions were genetically diverse (Loudet et al. 2003). The QTL analyses revealed four QTL for dry matter

content in nitrogen limiting conditions and nine QTL for amino acid content in high nitrogen conditions. For five out of seven traits studied by Loudet et al. (2003) more than seven QTLs were for mapped each. This is an impressive number as the most traits studied have revealed between two and four (Loudet et al. 2003).

The above example of the success of the Bay-0 x Shahdara population and the relevance to the study presented herein supported the choice of this RIL family as an ideal tool with which to dissect senescence.

1.4.1. QTL analysis

The principle of a QTL analysis is depicted in Figure 1.5. To summarise, if the genotype of related individuals is characterised (as in the Bay-0 x Shahdara population), the similarities in their phenotypes, relative to their genotypes, can be statistically compared. Therefore, the probability of a particular trait concurrently arising with a particular phenotype can be calculated to be either the result of inheritance of a specific genetic region (a locus) or chance. A log of odds (LOD) score is calculated for each marker, a score >2.4 is considered significant. A graph to show the location of significant peaks along each of the five chromosomes is then plotted and QTL involved in governing the trait of interest are revealed. The Bay-0 x Shahdara population is comprised of a large family of related individuals, of which 180 were used in the studies presented. This is a large population and therefore the chances of producing a positive correlation through chance not linkage, is less than 1/1000 (Collard et al. 2005).

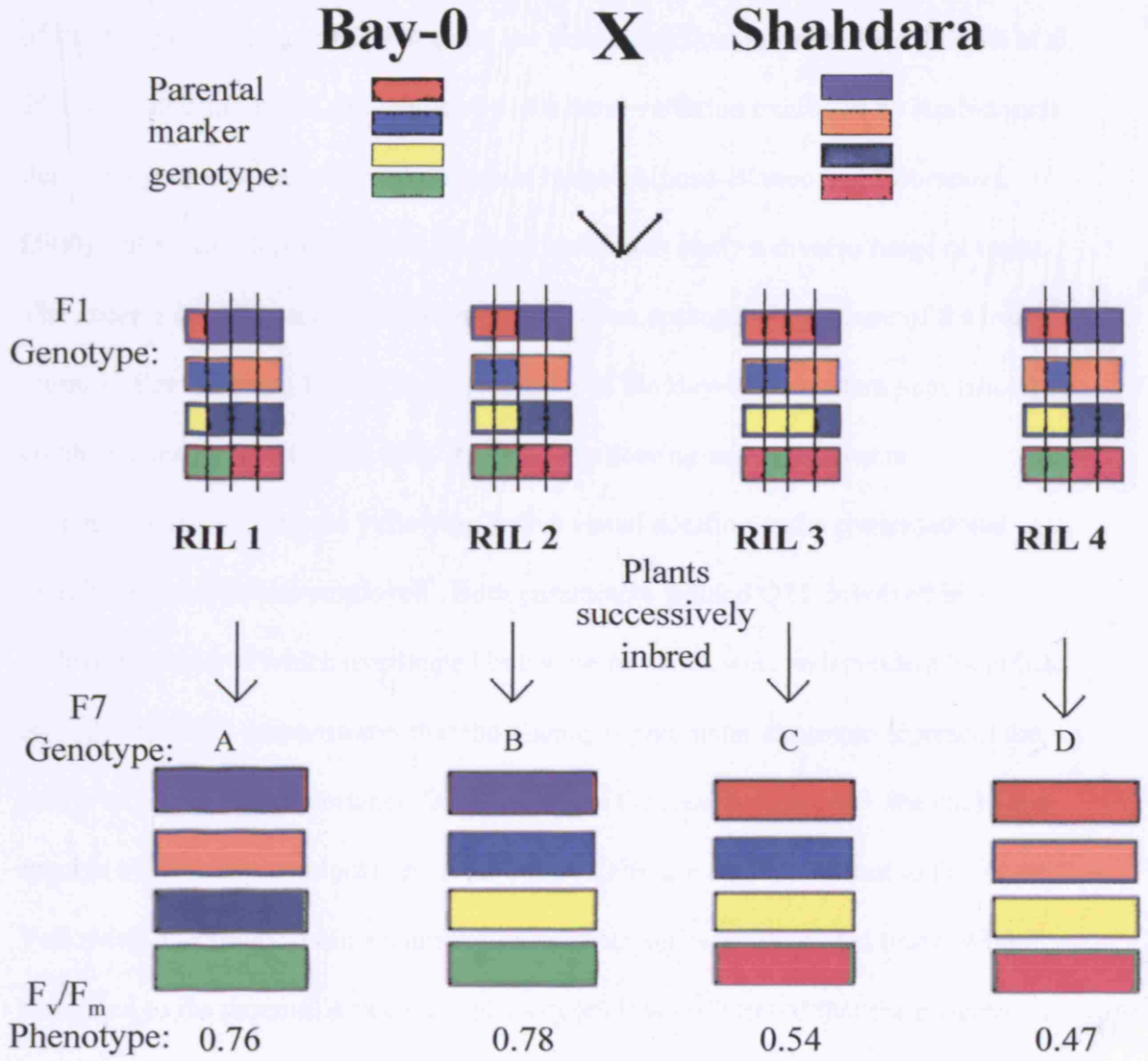


Figure 1.5. Depiction of the principle of quantitative trait loci (QTL) analysis.

The homozygous nature of the genome allows a measure of the trait of interest, such as maximum photosynthetic efficiency (F_v/F_m) as a measure of senescence, to be compared to the genotype. For example, RILs A and B have high F_v/F_m, the QTL analysis compares their marker genotypes to find out which chromosomal regions they share (e.g the purple band) and from this calculates where the genes controlling the trait may lie. Conversely, RILs C and D have a low F_v/F_m, therefore the red band may convey a more senescent phenotype.

Arabidopsis is unrivalled in its suitability for QTL analysis because of the availability of the complete genomic sequence and the dense genetic maps available (Loudet at al. 2003). Furthermore, the splendid array of natural variation exhibited by Arabidopsis due to its geographical and environmental range (Alonso-Blanco and Koornneef, 2000) make Arabidopsis an ideal plant with which to study a diverse range of traits. The success of QTL analysis relies on selecting an appropriate measure of the trait of interest. For example, Diaz et al. (2006) utilised the Bay-0 x Shahdara population to conduct a senescence related QTL study into yellowing and anthocyanin accumulation. To measure yellowing both a visual notation and a computational imaging technique was employed. Both parameters yielded QTL involved in yellowing, some of which overlapped but some of which were independent loci (Diaz et al. 2006). This demonstrates that the choice of parameter chosen to represent the trait is of paramount importance for the detail of the results obtained. The study and success of the study conducted by Diaz et al. (2006) are highly relevant to this study. Yellowing and anthocyanin accumulation are both senescence-related traits. When compared to the parental senescence phenotypes it was observed that the progeny exhibited transgressive segregation similar to that observed by Loudet et al. (2003) (Diaz et al. 2006). The QTL analysis revealed nine QTLs associated with leaf yellowing (YV/YP) and six involved in reddening (RV). The previous work by Loudet et al. (2003) allowed co localisations between these traits, nitrogen use efficiency and flowering time (Loudet et al. 2002a) to be correlated. It was therefore predicted that the use of QTL analysis to investigate glucose-induced senescence could also utilise these previous studies to build a more accurate and detailed picture of the senescence process.

1.5 Chlorophyll fluorescence

Maximum photosynthetic efficiency (F_v/F_m) was to be the principal measure of senescence used in this study as this method has proved itself to be an accurate and effective measure of senescence in previous studies (Pourtau et al. 2004; 2006; Wingler et al. 2004; Levey and Wingler, 2005).

Chlorophyll fluorescence has been established as an excellent method to measure plant performance in the field or laboratory. It is a non-destructive method that allows a fundamental feature of plant health to be measured throughout the life cycle. It can be carried out rapidly, increasing the replication size of trials and limiting experimental bias.

The principle of chlorophyll fluorescence is that an incoming photon that is absorbed by a chlorophyll molecule will undergo one of three possible fates. Firstly, it can be used to fuel photosynthesis, secondly it can be lost to the environment as heat or, thirdly, it can be lost as light energy in the process of fluorescence (Maxwell and Johnson, 2000). This latter pathway is utilized by fluorescence analysis. The three pathways are in competition for incoming photons, that is to say an increase in photochemistry, for example, will result in a decrease in fluorescence or heat loss. Therefore, the measurement of only one of the parameters can inadvertently reveal clues about the function of the others. Of particular interest for most physiological studies is the quantum efficiency of photosystem II as this is a good measure of health

and/ or developmental stage. The wavelength of light that has been fluoresced from a chlorophyll molecule is longer than that of absorbed light and so if light at a known wavelength is applied, the difference in wavelength and quantity of light that is re-emitted can be measured and converted in to a relative value as a measure of photosynthetic efficiency (Baker and Rosenqvist, 2004).

In the laboratory, plants are first dark adapted for approximately 20 minutes so that the initial acceptor, plastoquinone A (Q_A), is maximally oxidised rendering the photosystem II (PSII) reaction centres "open" and ready to accept electrons (Baker and Rosenqvist, 2004). Therefore, when dark adapted leaves are suddenly exposed to light, fluorescence is minimal, this is termed F_0. Once a PSII molecule has accepted an electron, however, it cannot accept a second until it has transferred the first to a downstream acceptor, such as plastoquinone B (Q_B). When a proportion of the reaction centres are closed due to this transferral time lag, fluorescence is increased as any subsequent incoming electrons cannot be absorbed and so are lost as heat or light (Maxwell and Johnson, 2000). The initial rise in fluorescence experienced in this stage is called the transient inflection (F_i) and it will continue to rise to the peak of fluorescence, point F_p (Baker and Rosenqvist, 2004). Following this point fluorescence begins to fall as enzymes that are involved in opening the stomata and carbon metabolism are activated by light and so the transferral rate of electrons through the PSII is increased. This phenomenon is termed photochemical quenching (*qP*) but the increase in rapidity of the process has a drawback in that more energy is converted to heat and lost to the environment, this is termed non-photochemical quenching (*NPQ*). This complete process is known as the Kautsky Effect (Kautsky et al. 1960).

In order to effectively compare values attained through fluorescence analysis the ratios of two fluorescence values are usually calculated. This is because the absolute values are the product of both the photochemical efficiency of the leaf and also its optical properties (Baker and Rosenqvist, 2004). The optical component must be removed from the calculation for accurate comparison of samples to be carried out.

Using a pulse-amplitude modulated (PAM) fluorometer, as used in this study, the minimum fluorescence (F_0) is first assessed by illuminating the leaf with a weak, modulated measuring light. At this point the all the reaction centres are open and fluorescence is minimised. The maximum fluorescence (F_m) - corresponding to the peak fluorescence (F_p) - is then determined by applying a saturating pulse of light. Due to the amplitude modulation of the measuring light, it is possible to differentiate between the chlorophyll fluorescence arising from the measuring light and any other external light source. The degree of variation (F_v = variable fluorescence) between the two points is then calculated by $F_m - F_0 = F_v$. From this the maximum photosynthetic efficiency of a particular plant or leaf can be calculated with the formula: F_v / F_m = maximum photosynthetic efficiency.

F_v/F_m has been widely used to measure compromised plant health that is not visible to the naked eye. For example in the monitoring of post harvest quality of lemon fruit that had damaged peel had a lower F_v/F_m than undamaged fruit. To the naked eye the damage only became apparent when some of the fruit developed mould several days later (Nedbal et al. 2000). This measurement has also effectively established spatial and developmental changes during senescence in a large number of studies (Wingler

et al. 2004; 2005; 2006; Pourtau et al. 2004; 2006; Levey and Wingler, 2005; Diaz et al. 2006; Araya et al. 2006; Masclaux-Daubresse et al. 2007). Furthermore, as senescence is a highly plastic trait involving many structural and physiological changes it was important to use a dynamic measurement. F_v/F_m proved its suitability in a study by Barbagello et al. (2003) testing the effect of different herbicides that were known not to directly affect photosynthetic metabolism in Arabidopsis. Six hours after treatment differences in the F_v/F_m phenotypes of plants treated with different herbicides was observed and after forty eight hours all treatments were significantly different. This example demonstrates that F_v/F_m is an excellent measure of metabolic perturbations. As this method was so well established as a measure of senescence the benefit for characterising plants and establishing a senescence response that may not be visible was apparent. To be able to monitor F_v/F_m in several plants at the same time, fluorescence imaging was applied using a kinetic imaging fluorometer based on the PAM technique (Nedbal et al. 2000).

1.6 Aims of the thesis

1) To demonstrate that the combination of low nitrogen and glucose uniquely induces senescence. That the phenotype of plants grown in these conditions cannot be mimicked by other combination of these two nutrients and is not stress induced.

2) To characterise the senescence phenotypes of members of the Bay-0 x Shahdara population. In doing this, it was intended to demonstrate that they display a range of responses to treatment and show transgressive segregation as observed in previous investigations (Loudet et al. 2002a; 2002b; 2003; Diaz et al. 2006).

3) To map loci specifically involved in the regulation of glucose-induced senescence and to investigate co-localisation with loci for other traits previously studied in this population.

4) To nominate candidate genes based on localisation and metabolic function for uncovered loci and their possible function and method of regulation of the senescence process.

5) To draw conclusions on the role and mechanisms that glucose and nitrogen interactions have on the regulation of developmental senescence.

Chapter 2

Materials and methods

2.1 Plant material

Arabidopsis thaliana (L.) Heynh. Col-0 was obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK). The Bay-0 x Shahdara population (Loudet et al. 2002) was obtained from INRA Versailles (INRA, Versailles, France). F7 seeds obtained from single seed descent were used. The use of the selected recombinant inbred lines (RILs), 232, 272, 310, 45, 83 and parental accessions Bay-0 and Shahdara were chosen on the basis of their contrasting senescence phenotypes (Diaz et al. 2005).

Near isogenic lines (NILs) were provided by INRA Versailles. NILs were generated as heterozygous inbred family (HIF) lines by segregating the Bay-0 and Shahdara alleles (Loudet at al. 2005) between the molecular markers NGA8 and MSAT 4.8 situated at the top of chromosome 4. From the physical map designed by Loudet et al. (2002) it was possible to identify RILs showing residual heterozygosity within this region in the F6 generation. Seeds were planted from the F6 plants that had the Bay-0 allele (NIL 145 Bay-0 and NIL 312 Bay-0) and Shahdara allele (NIL 145 Shahdara and NIL 312 Shahdara) fixed in this position for the segregating markers. From the resulting F7 progeny 24 plants from each NIL were screened to ensure the expected allele was present

at the locus. The expected frequency of ¼ homozygous with Bay-0 alleles at the locus, ¼ homozygous for Shahdara and ½ heterozygous was observed (Masclaux-Daubresse, personal communication). F8 seeds of these two pairs of lines were then used for the subsequent experiments.

The freezing-sensitive *sfr6* mutant was obtained from Professor Marc Knight (Durham University). The lines were generated by Warren et al. (1996) from the pedigreed mutant set of James and Dooner (1990), derived from Col-0.

2.2 Growth conditions

All plants were grown in a JUMO ILP F200 growth chamber (Snijders Scientific, The Netherlands) under controlled conditions and cultivated on agar. For high nitrogen (HN) medium, half-strength Murashige and Skoog (MS) basal salt mixture (M 5524: Sigma Aldrich, Gillingham, Dorset, UK) containing 30 mM nitrogen (10.3 mM NH_4^+ and 19.7 mM NO_3^-) was used. For low nitrogen (LN) medium, quarter strength Murashige and Skoog (MS) basal salt mixture without NH_4NO_3 (M 2909: Sigma Aldrich) was used to lower the nitrogen concentration to 4.7 mM. The pH of the nutrient solution was adjusted to 5.6-5.8 with KOH. Ten grams of agar per 1 litre of nutrient solution (1%) was added prior to sterilisation. Autoclave conditions for all media were 121°C, 15 pounds per square inch (p.s.i.) pressure for 20 minutes on a liquid cycle. Nutrient was allowed to cool to approximately 50°C before being poured in to sterile petri dishes in a

laminar flow hood. Plates were left to solidify for approximately 60 minutes and then stored at 4 °C.

To test the effect of different sugars on the RILs, low nitrogen medium was prepared and autoclaved. To prevent sucrose hydrolysing to glucose and fructose in the autoclave the different sugars were aseptically added afterwards to the cooled (approximately 50°C) medium by sterilisation through a 0.22µm Millipore membrane (Millipore Corporation, Bedford, Massachusetts, USA). Medium was then evenly distributed in to sterile petri dishes (approximately 25 ml per dish). The concentration of fructose and glucose was 2% w/v (111 mM), whilst the sucrose concentration was 55.6 mM, assuming that it may be hydrolysed into two hexose molecules. For all other glucose containing media 2% (w/v) glucose (111 mM) was added prior to sterilisation.

Media containing mannitol and sorbitol contained 2.02% w/v of each osmolyte to maintain a concentration, the same as glucose medium, of 111 mM. Each was added to the LN medium and the medium was pH adjusted and autoclave sterilised as previously described.

Seeds were sterilised in commercial bleach and then washed three times in sterilised water. Seeds were then re-suspended in sterilised 0.7% low melting-point agarose and pipetted on to the agar medium in the petri dishes. Approximately five plants were cultivated per dish. The plates were sealed using Micropore surgical tape (M 1530-0: Micropore, 3M Health Care, St. Paul, Minnesota, USA) and left to stratify at 4 °C for 3

days. The plates were then transferred to growth cabinets and grown at vertical orientation at a photon flux density of 100 μmol m^{-2} s^{-1} for 16 hours per day at a day time temperature of 22 °C and a night time temperature of 18 °C.

2.3 Maximum photosynthetic efficiency (F_v/F_m)

Maximum photosynthetic efficiency (F_v/F_m) was measured using a pulse-modulated imaging fluorometer (Fluorcam 700MF; Photon Systems Instrument, Brno, Czech Republic). Plants were dark adapted for 20 minutes and then the minimum fluorescence (F_0) was measured by exposing the plants to a modulated red light. A flash of saturating white light was then administered (0.8 s duration) and maximum fluorescence (F_m) was determined. To calculate the maximum photosynthetic efficiency (F_v/F_m) of the entire leaf rosette the formula $(F_m - F_0)/F_m$ was applied (Krause and Weis et al. 1983). For each experiment a minimum of five petri dishes per line per treatment was used, each with a minimum of five plants per petri dish, resulting in a minimum of 25 plants per line per treatment.

2.4 Chlorophyll content

Samples were washed in distilled water and the root material was removed. The weight of harvested leaf rosettes was determined and then 1 ml of 80% acetone was applied to

each sample in an eppendorf tube. The samples were ground within the tube using a small plastic pestle and left on ice. The acetone containing the dissolved chlorophyll was then centrifuged for 2 minutes transferred to a new eppendorf tube. Chlorophyll a and b content was determined by measuring absorbance at 663 nm (chlorophyll a) and 646 nm (chlorophyll b) in a glass cuvette in a spectrophotometer (Ultraspec 3100 *pro*, Amersham Biosciences). The "blank" reading of 80% acetone was established, before 1 ml of each extract was measured at the same wavelengths. The concentration of chlorophyll a and b was determined with the following equations (Lichtenthaler and Wellburn, 1983):

$$12.21 * (A663\text{-}blank663) - 2.81 * (A646\text{-}blank646) = Chl\ a\ \mu g\ ml^{-1}$$

$$20.31 * (A646\text{-}blank646) - 5.03 * (A663\text{-}blank663) = Chl\ b\ \mu g\ ml^{-1}$$

Ratios were determined by dividing Chlorophyll a / Chlorophyll b.

2.5 Sugar contents

2.5.1. Extraction of sugars

Sugar contents were determined enzymatically according to Stitt et al (1989). Plants were harvested approximately 8 hours into the photoperiod. Root material was removed and plants were washed thoroughly in distilled water. Plant material was weighed and applied to a pre-weighed eppendorf tube containing 1 ml of 80% ethanol heated to 80°C. The samples were left for 12 minutes until the chlorophyll had been extracted. The

ethanol was then transferred to a second (pre weighed) eppendorf tube. The remaining plant material was washed with 0.5 ml of 80% ethanol and then combined with the first 1 ml of extract. Both the tube containing plant material and the tube containing the combined ethanol and extracts were dried over night in a freeze dryer (Edwards Freeze Dryer Modulyo. D.Bewhey Ltd, High Canons, Well End, Boreham Wood, Herts. UK). The dried tubes were then weighed and the dry weights of the samples determined. The dried extract was then dissolved in 200 µl of distilled water and, if not used directly, frozen at -20°C for future use.

2.5.2. Determination of sugar contents

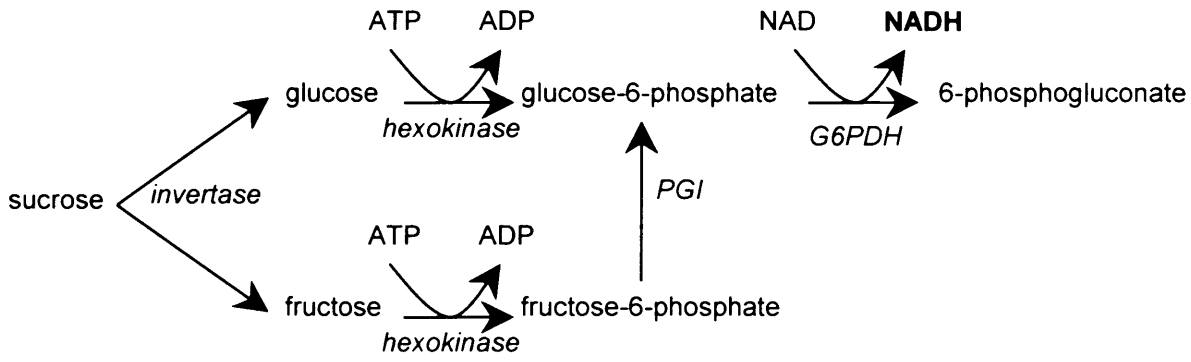
A solution of HEPES buffer was made containing 100mM HEPES/5 mM $MgCl_2$ (pH 7.5). NAD (40 mM) and and ATP (100 mM) were dissolved in this buffer.

Four sugar phosphorylating enzymes were diluted as follows:

- Glucose-6-phosphate dehydrogenase (G6PDH) (Roche 165875; 1000U/ml), 1:20 in HEPES buffer.
- Hexokinase (Roche 1426362; 1500 U/ml), 1:30 in HEPES buffer.
- Phosphoglucose-isomerase (PGI) (Roche 127396; 2 mg/ml), 1:10 in HEPES buffer.
- Invertase (Sigma I4504; 355 U/mg) 1 mg/150 µl in HEPES buffer.

The sugar contents were then determined using an ELX 808UI microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Into each well of the microplate the following

was added: 200 µl HEPES/ 5 mM $MgCl_2$ (pH 7.5), 10 µl 40 mM NAD, 10 µl 100 mM ATP, 10 µl G6PDH and 10 µl of either distilled water (control sample), standard sugar solution or sample. After the initial readings at 340 nm had been taken, the three enzymes were added in order of: 10 µl Hexokinase to assess glucose concentration, 10 µl PGI to assess fructose and finally 10 µl invertase for sucrose concentration. The reaction was monitored by recording the absorbance of NADH at 340 nm. Each reading took 15 minutes to allow the reaction to occur. After the kinetics had ceased the initial starting absorbance value was subtracted from the final absorbance value to give a numeric value of the increase in NADH concentration, measured at 340 nm. The final value at each step was then used as the initial starting value for the subsequent step. The diagram below summarises the reactions:



Standard sugar solutions were prepared and diluted in a two fold dilution series ranging from 50 nmol hexose/ 10 µl H_2O – 3.125 nmol hexose/ 10µl. The relationship between the standard sugars and absorbance was then evaluated as a measure of absorbance change per nmol of hexose at 340 nm. The values of absorbance were then plotted on a graph and a factor F of a linear regression determined based on the angle of incline. The

F values for fructose, glucose and sucrose were calculated to be 0.0145, 0.0168 and 0.0126 absorbance units/nmol hexose, respectively (Figure 2.1). The absorbance of the samples taken from the RILs could then be compared to the standard to calculate their contents. From this the formula used for total sugars was:

(absorbance units/ F value)/ (mg fresh weight / dilution factor) = nmol / mg fresh weight.

All sucrose contents are expressed as hexose values.

For each sample, three replicate reactions were measured.

2.6 Reverse transcriptase polymerase chain reaction (RT-PCR)

2.6.1. RNA extraction and cDNA synthesis

Plant material was harvested approximately 8 hours into the photoperiod after growth on agar medium for 30 days and was then flash frozen in liquid nitrogen. Frozen plant material was then transferred to lysing matrix D tube (BIO 101 Systems; Q BIOgene) and RNA was extracted by homogenizing the plant material in TRIzol reagent (Invitrogen 15596-018) using a FP220 ribolyser (Q-Biogene). RNA was isolated by phenol-chloroform precipitation then washed in 80% ethanol. The resulting pellet was then dried for 10 minutes at 90°C before being dissolved in 150 µl of distilled H_2O. The quantity of RNA extracted was then calculated by reading the samples' absorbance at 260 nm with a photometer (WPA Biotach Photometer UV 1101. Jencons PLS.). RNA was then frozen at -80°C.

First strand cDNA was synthesized with Omniscript Reverse Transcription Kit (Qiagen, Crawley, UK) with random primers p(dN)6 and oligo (dT) (Roche Diagnostics). For each sample the following quantities were used:

- 10x Buffer RT 2 µl
- dNTP Mix (5 mM each dNTP) 2 µl
- Oligo-dT primer (10 µM) 2 µl
- Random primers p(dN)6 2 µl
- RNase inhibitor (10 units/ µl) 1µl
- Omniscript reverse transcriptase 1 µl
- Template RNA Variable

The quantity of template RNA required was 1 µg for the cDNA synthesis. This was calculated with the following formula:

Absorbance at 260 nm x 40 x 201 (5 µl RNA in 1 ml H_2O) = Quantity RNA µg/ml

1000 / Quantity RNA (µg/ml) = µl of RNA required for cDNA synthesis (1µg).

A separate reaction containing no Omniscript reverse transcriptase was carried out for each sample. This ensured that observed results were related to mRNA content and not genomic DNA contamination.

2.6.2. Polymerase chain reaction conditions

A polymerase chain reaction (PCR) master mix was made containing, per reaction:

- 10 x Thermal buffer (B9004S) 2.5 µl
- dNTP Nucleotide solution (N0447S) 0.5 µl
- Taq DNA Polymerase (M0267S) 0.1 µl
- Distilled H_2O 20.4 µl

All the components of the mastermix were purchased from New England BioLabs. The components of the mix were all added to an eppendorf tube except the Taq polymerase. The mastermix was kept on ice whilst 0.5 µl of both forward and reverse primer and 0.5 µl of cDNA was added to a PCR tube per reaction. The Taq polymerase was then added to the mastermix and mixed by pipetting. The mastermix was vortexed for a few seconds and then 23.5 µl was added to each of the PCR tubes. The tubes were then centrifuged for 10 seconds to collect residual mix off the sides of the tubes and were put in to a Techne Genius (Jencons - PLS) PCR machine.

The PCR conditions were 5 minutes at 94°C, followed by cycles of 30 second at 94°C, 30 seconds at 55°C and 45 seconds at 72°C with a final extension step of 5 minutes at 72°C. The primers used are listed in Table 2.1. The RT-PCR results were confirmed at least once with independently synthesised cDNA.

2.6.3. Gel electrophoresis

Fifty ml of a 1% (w/v) agarose gel was prepared by adding 0.5 g agarose (type II, Sigma-Aldrich) to 50 ml TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) in a 100 ml conical flask and melted in a microwave on a medium setting. Gels were poured in to a level platform fitted with a comb and left to set.

Droplets of 3 µl of 6x blue loading dye (Fermentas), one per sample, were pipetted on to a strip of parafilm then 15 µl of each of the PCR products was added to the drop. The total quantity of 18 µl per sample was then pipetted in to a well of the gel. Gels were run in an electrophoresis cell (Embi Tec RunOne Electrophoesis Cell) at 100 volts for approximately 25 minutes, or until the loading dye indicated that the sample had migrated ¾ of the way across the gel. Gels were then removed and stained for 15 minutes with a 1 µg/ml ethidium bromide solution before imaging with a UVP White / UV Transilluminator imager and camera. Images were printed with a video copy processor (Mitsubishi P93).

2.7 Broad sense heritability

The broad sense heritability (H) was calculated as the ratio between genetic variance and total phenotypic variance (Loudet et al. 2002). An analysis of variance model (ANOVA) was performed on the F_v/F_m data, from this the following calculation was carried out:

(Between group variance – within group variance) = A

A / number of replicates = B

B / (B + within group variance) = H

2.8 Quantitative trait loci analysis

The QTL analyses were carried out using QTL cartographer 1.14 (Basten et al. 1994; 2000) supplied by http://stagen.ncsu.edu/qtlcart/cartographer.html. The mean values of the maximum photosynthetic efficiency F_v/F_m for each RIL were used for the QTL analyses. Simple interval mapping was first used and Composite Interval Mapping (CIM) was then carried out using Model 6 of Windows QTL Cartographer version 2.5. A logarithm of odds (LOD) score was estimated for the trait from 1000 permuation tests with an overall error level of 5% (Churchill and Doerge, 1994). From the CIM results the additive effect of each detected QTL and the mean effect of the replacement of both Shahdara alleles by Bay-0 alleles at each locus was calculated (Diaz et al. 2005). The R^2 value was calculated by variance analysis by Windows QTL Cartographer. This represents the overall contribution to total phenotypic effect attributed to each locus, by

variance component analysis. The model is used to calculate the value involved using the genotype at the nearest marker and the detected QTL as random factors in an ANOVA. The analysis of the subsequent ANOVA was carried out using *lm*() and *aov*() functions of statistical package, S-PLUS 3.4 (Statistical Sciences, Inc; Loudet et al. 2002a; Masclaux-Daubresse; personal communication).

2.9 Chromosome diagrams of least senescent recombinant inbred lines (RILs)

Chromosome diagrams were obtained from online graphics software, Graphical GenoType software (http:/www.dpw.wau.nl/pv/pub/ggt/). Marker data for the Bay-0 x Shahdara was plotted to show the contribution of each parental allele on each of the five chromosomes for the ten recombinant lines with the highest F_v/F_m at the latest time point. Chromosome IV was then selected and cut for each of the lines and presented together in Microsoft PowerPoint 2000.

2.10 Candidate gene nomination

The TAIR website (www.arabidopsis.org) was used to identify genes that lie between marker genes NGA8 and MSAT 4.8. Candidate genes were nominated based on their co-

localisation to the uncovered locus on chromosome IV. Genes were selected that were involved in senescence, cold tolerance and flowering.

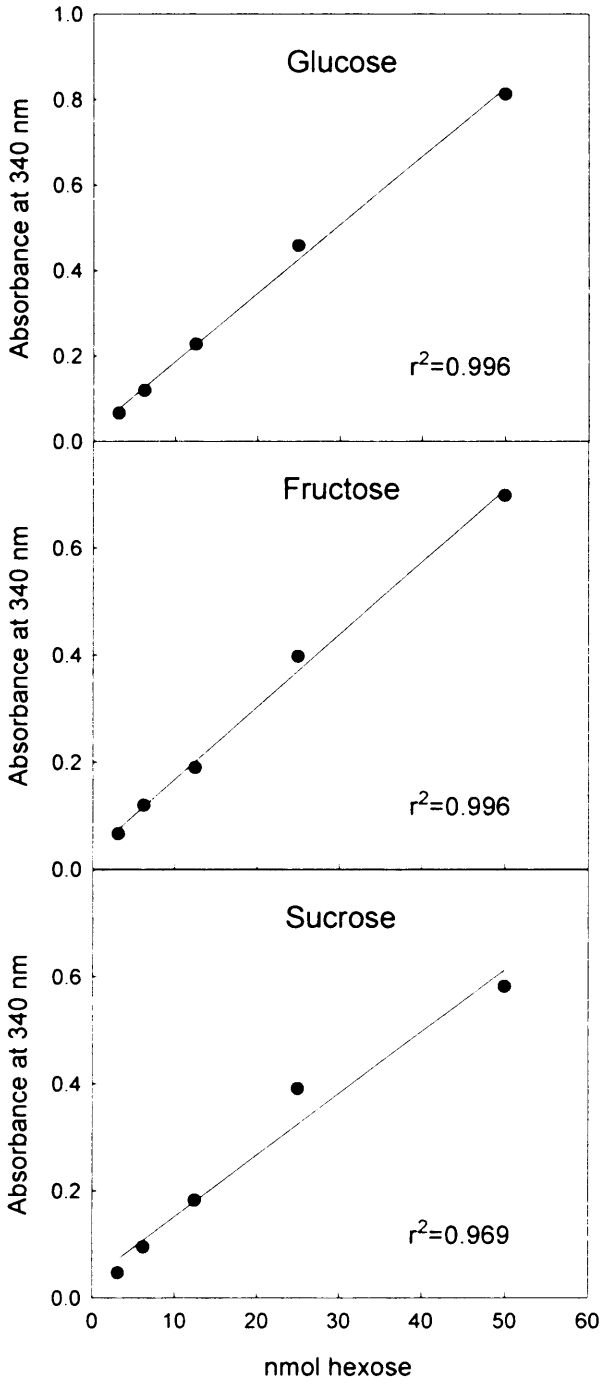


Figure 2.1. Linear regression of sugar standards.

Average values for the absorbance of glucose, fructose and sucrose through a series of two fold dilutions. The angle of incline (Y) was then calculated to give the value of absorbance units/ nmol sugar.

AGI Code	Gene name	Forward primer sequence	Reverse primer sequence	Product size
At3g18780	*ACT2*	GACGTGACCTTACTGATTACCTC	TATCCACATCACACTTCATGATT	321
At5g35630	*GS2*	GACATTTCAGATGCTCATTACAA	TAACCATGAAAATCTATCCCTGT	396
At5g65010	*ASN2*	ACCACGAGTTTCAGTTTACAGTT	GCAGAAGTTGATTTGTTAGCTCT	301
At2g42530	*COR15b*	TGTTGGTACCGTCAGAGTTG	AAAGCTTTCTTAGCTTCCTCAGT	252
At1g72610	*GER1*	CAGCTAGACTTGACTTAGCTCCT	GAGTTCAGTGGGAAGACTGTTAG	299
At5g45890	*SAG12*	AGGAGCAACACAAATAAAGAAAG	ACGAATAGAATTGGAAATCAAAA	335
At1g66390	*PAP2*	GATAAGTATGGAGAAGGCAAATG	GAGGGGAAATAATGTTTTTCTTT	313
At2g39330	*jacalin*	CTACTCCGACTCCTATAATTCCA	AGATAATCATTGGTGTCTCGACT	300

Table 2.1. Gene names and primer sequences used to test gene expression by RT-PCR.

Chapter 3

Regulation of leaf senescence by sugar and nitrogen in different accessions of Arabidopsis

3.1 Introduction

The onset and progression of senescence occurs via complex nutrient signalling pathways. Numerous studies have demonstrated that sugars and nitrogen are essential signal components in these cascade reactions (Price et al. 2004). Although both nitrogen and glucose sensing and/or signalling mechanisms may be initiated independently, the pathways are very closely linked and changes in one will be sensed and alter the fate of the other. This interaction is called "cross-talk" and is thought to occur by the sensing of either the carbon:nitrogen (C:N) ratio (Coruzzi and Zhou, 2001) or the sink-source balance between spatially separated organs or tissues.

Nitrogen often absorbed from the soil in the form of inorganic NO_3^- (Marschner, 1995), has also been shown to regulate gene expression, particularly genes involved in nitrogen assimilation and utilisation (Forde, 2002; Stitt et al. 2002; Price et al. 2004; Martin et al. 2005). Price et al. (2004) conducted a micro-array study to observe gene expression in response to treatment with nitrogen. The group found that 106 genes were up-regulated and 129 were repressed. Predominantly to be up-regulated were genes involved in nitrate/nitrite assimilation, such as nitrate reductase 1 (*NIA1*) and ammonium assimilation such as asparagine synthetase (*ASN2*) (Price et al. 2004). The

results cited above had been largely observed previously by Wang, et al. (2000) however this group also reported the response of genes previously unknown to respond to nitrate treatment including *CALCIUM ANTIPORTER1* (*CAX1*), a MYB transcription factor (Wang et al. 2000).

Previous studies have demonstrated that high levels of glucose within leaf cells repress genes involved in photosynthesis and activate genes involved in storage (Jang and Sheen, 1997; Lalonde et al. 1999) and also affect the transcription of genes involved in nitrogen metabolism (Masclaux et al. 2000; Price et al. 2004; Pourtau et al. 2006). The internal regulation of nitrogen and carbon levels by sugar sensing has been divided in to two categories based on carbohydrate availability. The first, known as the "feast" response is instigated when carbohydrate levels are high and results in the up-regulation of genes involved in nitrogen assimilation and starch synthesis. The second category, the "famine" response, is instigated by low carbohydrate and results in the expression of genes involved in photosynthesis and the remobilisation of nutrients such as nitrogen (Lalonde et al. 1999). From reports such as this it appears that the relationship between nitrogen and sugars is synergistic, but other studies have also uncovered antagonistic interactions between these two essential nutrients. For example, it has been identified that glucose can cause the down-regulation of an asparagine synthetase gene (*ASN1*), which is involved in the transport and storage of nitrogen (Lam et al. 1998; Price et al. 2004). This exemplifies that the relationship between these two essential nutrients is complex but it also indicates that sugars may regulate the balance to a greater degree than nitrogen. This has been recurrently demonstrated in a number of studies. For example, Masclaux et al. (2000) identified that the youngest, top-most, leaves of tobacco

contained higher levels of ammonium and nitrate than the older, middle leaves. The oldest, most senescent leaves also contained high levels of ammonia, similar to that of the youngest, but contained low levels of nitrate. Conversely, sugar levels in the respective leaves showed the opposite result, in that the youngest leaves had the lowest sugar levels compared to the older, middle leaves. The authors of this study concluded that the low assimilation of ammonia in the youngest leaves was probably due to carbohydrate limitation (Masclaux et al. 2000). Additionally, the study conducted by Price et al. (2004) not only that uncovered gene regulation by nitrate and ammonia but also that a number of genes that had been previously cited as nitrate responsive were actually only expressed in the presence of both nitrogen and sugar. This clearly demonstrates that the interactions between sugar and nitrogen play an important role in gene regulation.

In this study the relationship between sugar and nitrogen was investigated by comparing the senescence phenotype of a selection of recombinant inbred lines (RILs) and their parental accessions. The recombinant inbred population chosen for the study was the Bay-0 x Shahdara population which is a relatively new population (Loudet at al. 2002a). It was therefore important to establish that this family would be a suitable tool with which to investigate senescence and also that the chosen parameters were effective measures of senescence in the RILs. The plants were treated either with high nitrogen (HN), high nitrogen plus 2% glucose (HNG), low nitrogen (LN) or low nitrogen plus 2% glucose (LNG) in the growth media. The measured parameter was maximum photosynthetic efficiency (F_v/F_m).

Maximum photosynthetic efficiency (F_v/F_m) was chosen because it is a non-destructive method that has been shown to be effective in monitoring senescence in other accessions such as Col-0 (Wingler et al. 2004), Ws-2 and Ler-0 (Pourtau et al. 2004).

Previous studies had shown that glucose in combination with low nitrogen (LNG) accelerated senescence (Pourtau et al. 2004; 2006; Diaz et al. 2005), however, it was not known whether other sugars, specifically sucrose and fructose, could mimic this response. Glucose is the most readily metabolised sugar and high amount of glucose may cause the repression of genes involved in utilizing sucrose in yeast (Lalonde et al. 1999). Furthermore, high levels of sucrose in sugarbeet (*Beta vugaris)* results in a decrease in sucrose transport but high levels of glucose or fructose affect transport to a much lesser extent (Chiou and Bush, 1998). Establishing whether the role of glucose in regulating senescence is unique was therefore essential before postulations about possible mechanisms could be justifiably made.

As the rationale for this study was that senescence is regulated by the interactions of sugar and nitrogen, it was important to ensure that observed responses were the result of signalling and/or metabolism and not an osmotic stress response. Many studies have already demonstrated that glucose and osmolyte response pathways are separate. For example, Arroyo et al. (2003) conducted a study in which mutant Arabidopsis seedlings, *abscisic acid insensitive 4* (*abi-4*), *abscisic acid insensitive 5* (*abi-5*) and *constitutive triple response* (*ctr-1*), were grown in combination with 7% w/v glucose or mannitol. Analyses of the corresponding transcripts were conducted using RT-PCR and northern blotting. The results showed that although mannitol could induce

ABI4 and *CTR1* expression, induction was significantly lower than in glucose treatments. The expression of *ABI5* was inconclusive when analysed only with RT-PCR and northern blot hybridisation but was confirmed by treating plants with 2-deoxyglucose (2DG), a phosphorylable glucose analogue which has been shown to mimic glucose in the regulation of specific genes but is used in too small concentrations to instigate an osmotic effect (Arroyo et al. 2003; Jang and Sheen, 1994).

To establish that results obtained in my own studies were due to the effect of glucose and nitrogen on gene action and/or metabolism, not osmotic stress, glucose was compared as a treatment to two known osmolytes, mannitol and sorbitol and growth and development of *Arabidopsis* accession Col-0, was observed.

3.2 Results

3.2.1. The effect of glucose, mannitol and sorbitol on maximum photosynthetic efficiency (F_v/F_m)

Mannitol and sorbitol are known to exert an osmotic effect on plant growth and development. To determine that observed results from glucose and nitrogen treatments on senescence was sugar specific, maximum photosynthetic efficiency (F_v/F_m) in Arabidopsis Col-0 was measured. Mannitol, sorbitol and glucose were all used in the same concentrations (111 mM), corresponding to 2% (w/v) glucose.

The maximum photosynthetic efficiency (F_v/F_m) showed a significant difference between the treatments at 40 days after planting (DAP) when glucose-treated plants (LNG medium) showed a rapid and progressive decline compared to mannitol (LNM), sorbitol (LNS) or without the addition of an osmolyte (LN) (Figure 3.1). An ANOVA revealed a highly significant difference between treatments ($P = 0.000$) and a Tukey test revealed a significant difference between LNG and the other treatments at DAP 49.

3.2.2. Maximum photosynthetic efficiency (F_v/F_m) for selected RILs in response to treatment with nitrogen and sugar

Maximum photosynthetic efficiency (F_v/F_m) values were similar for all lines in treatments in high nitrogen (HN) and high nitrogen + 2% glucose (HNG) (Figure 3.2).

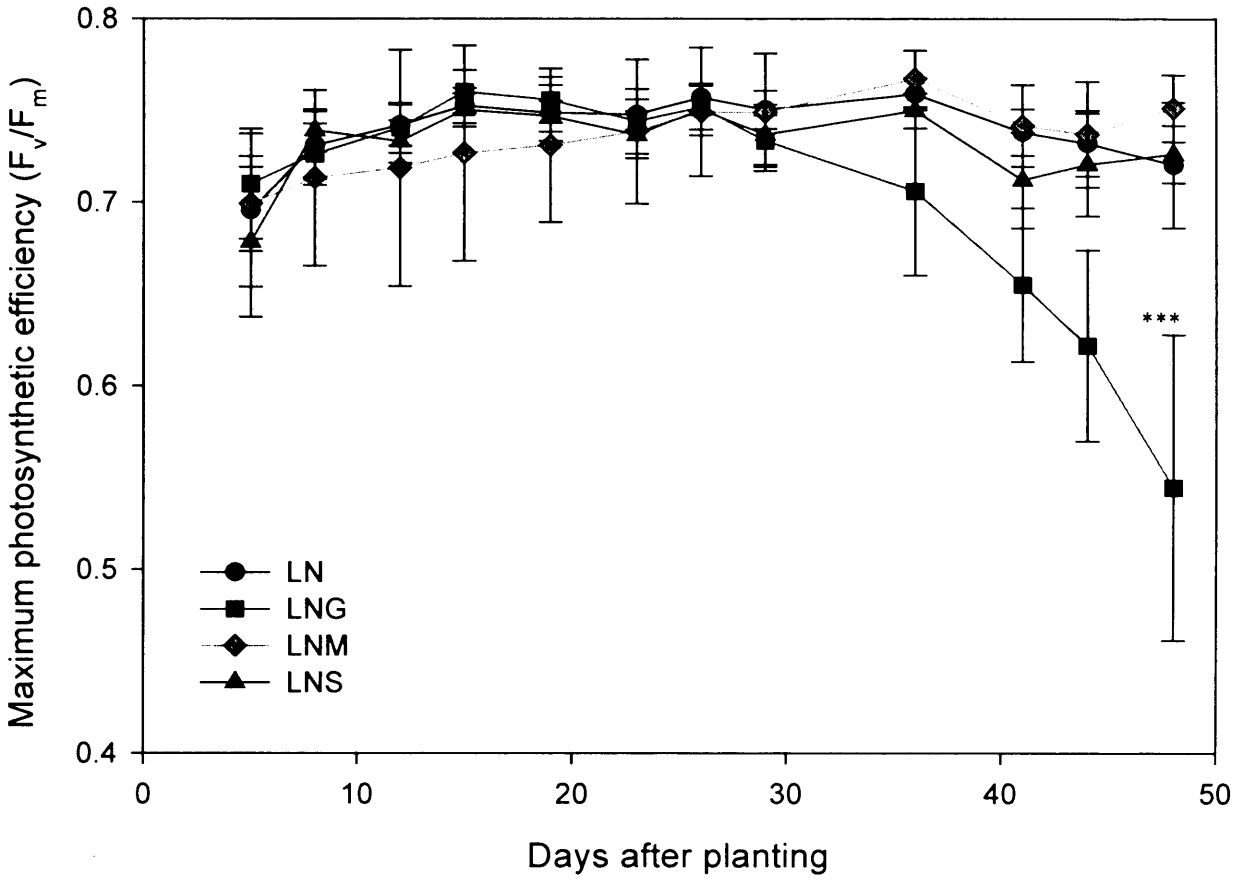


Figure 3.1. Maximum photosynthetic efficiency of Col-0 plants grown in combination of low nitrogen (LN) and osmolytes glucose (LNG), mannitol (LNM) and sorbitol (LNS).

Data is a means of a minimum of 25 plants per treatment. Error bars denote standard deviation. *** ANOVA test comparing treatments at DAP 49 revealed P = 0.000 and a Tukey test revealed that LNG is the only significantly different treatment at DAP 49.

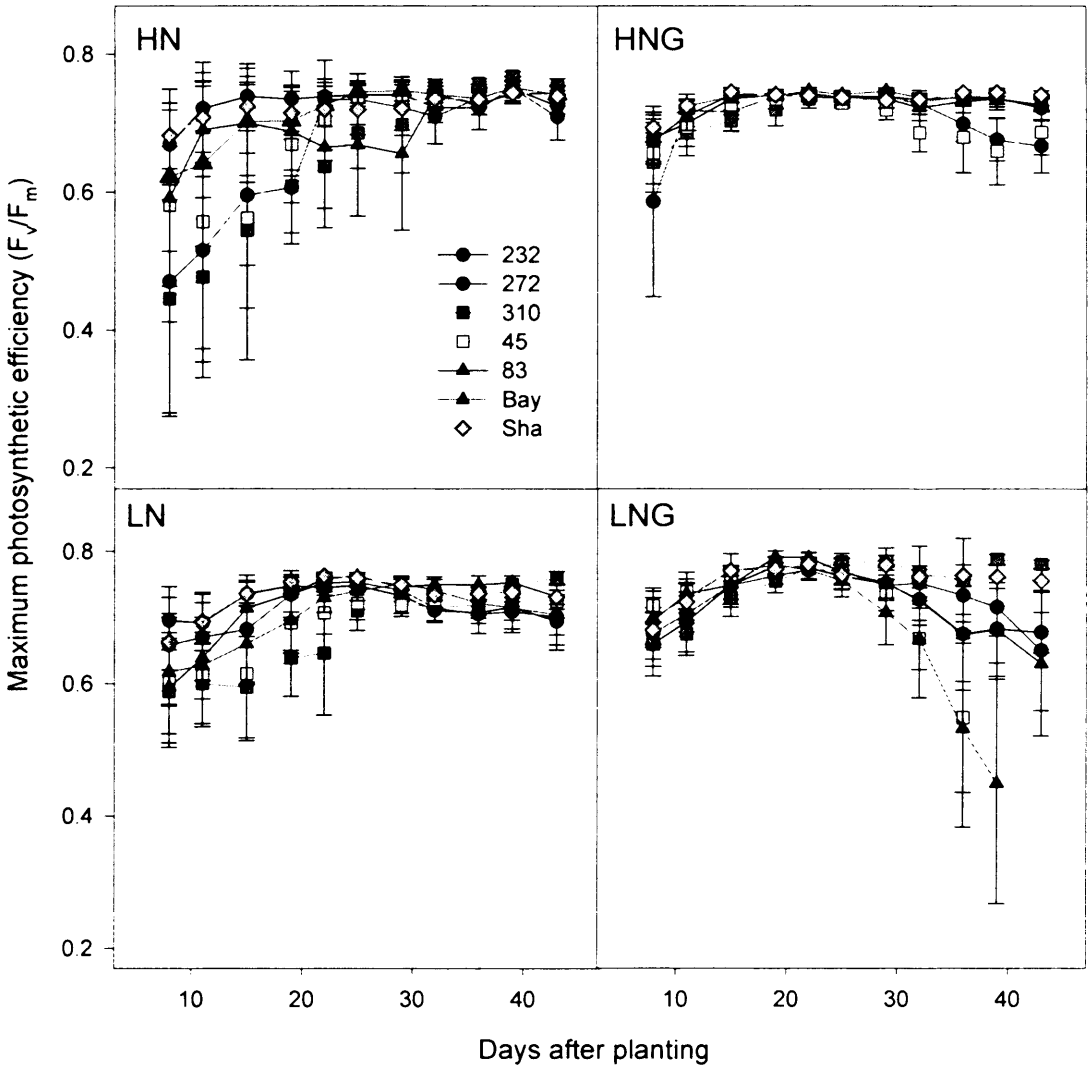


Figure 3.2. Maximum photosynthetic efficiency (F_v/F_m) of selected recombinant inbred lines and their parental accessions in response to treatment with nitrogen and glucose.

Data are a means of a minimum of 25 plants per RIL and error bars denote standard deviation. HN = high nitrogen, HNG = high nitrogen plus glucose, LN = low nitrogen, LNG = Low nitrogen plus glucose.

F_v/F_m remained high throughout the experiment, a slight decline was observed in all lines, except 310, after DAP 40 in both treatments.

In low nitrogen (LN) conditions all the lines had very similar F_v/F_m values throughout the trial. No differences between the RILs and their parental accessions were observed until after DAP 40 and even then, only line 310 was noticeably different because it retained a high F_v/F_m rather than starting to decline.

The most striking response to treatment was observed in low nitrogen plus 2% glucose (LNG) grown plants, where all lines showed an accelerated senescence phenotype compared to treatments LN, HN and HNG. All lines, except 310 and Shahdara, had started to senesce by DAP 35 and the F_v/F_m of RILs 45 and 83 had started to decline as early as DAP 25.

3.2.3. Maximum photosynthetic efficiency (F_v/F_m) and development of selected RILs in response to treatment with different sugars.

In all treatments the most rapid decline in F_v/F_m was observed in RIL 45, conversely in all treatments RIL 310 maintained a high F_v/F_m throughout the trial and RIL 232 displayed an intermediate phenotype in all treatments (Figure 3.3). All three of the sugar treatments showed similar response patterns in all the RILs and their parental accessions, in that the F_v/F_m of most lines had started to decline after DAP 25 and development patterns of the different lines were similar between sugar treatments.

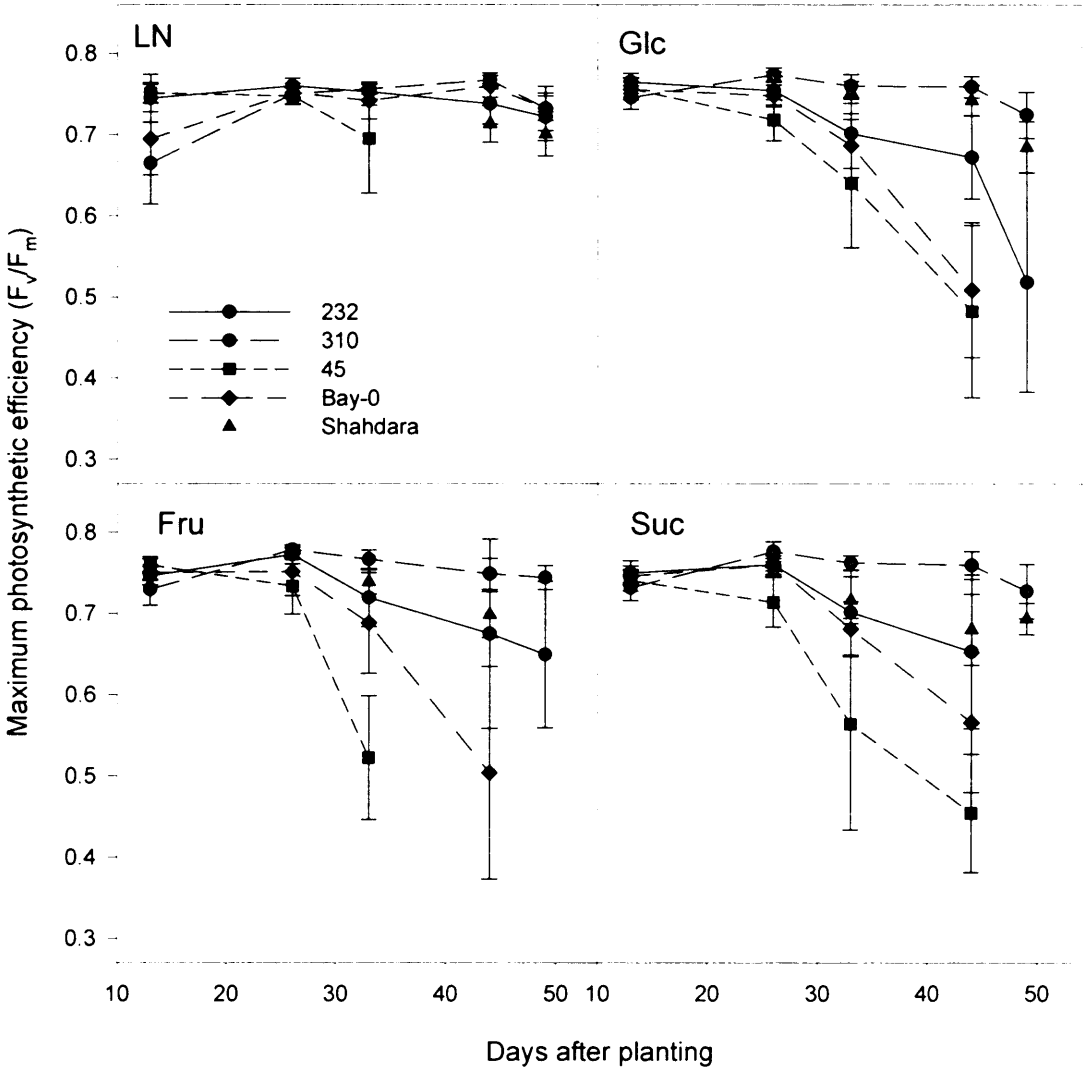


Figure 3.3. Maximum photosynthetic efficiency (F_v/F_m) of recombinant inbred lines (RILs) and their parental accessions when grown in combination of low nitrogen and different sugars.

Data are the means of a minimum of 25 plants per RIL, error bars denote standard deviation.

Only a few plants of RIL 310 bolted and, subsequently flowered, in LNG and LNS treatments (Figures 3.4 and 3.5) and no bolting was observed on LNF or without sugar. Bolting and flowering was accelerated in all sugar treatments in Bay-0 compared to LN. The bolting and flowering phenotype of Bay-0 and 45 was identical in LNF and LNS treatments. Shahdara never achieved 100% bolting plants in either LNF or LNG.

The most striking feature of the senescence phenotype was that neither of the parental accessions, Bay-0 or Shahdara, had >40% senescent plants at the end of the trial when treated with LN (Figure 3.6). In sugar treatments, however, senescence was accelerated (by comparison) in both parental accessions, particularly Bay-0. An apparent discrepancy between the F_v/F_m phenotype and the visible senescence was revealed in that RIL 310 showed 100% senescent plants by DAP 33 in all treatments, but retained a high F_v/F_m throughout the experiment. This is because this line showed signs of senescence in its oldest leaves but then produced new leaves at the top of the rosette.

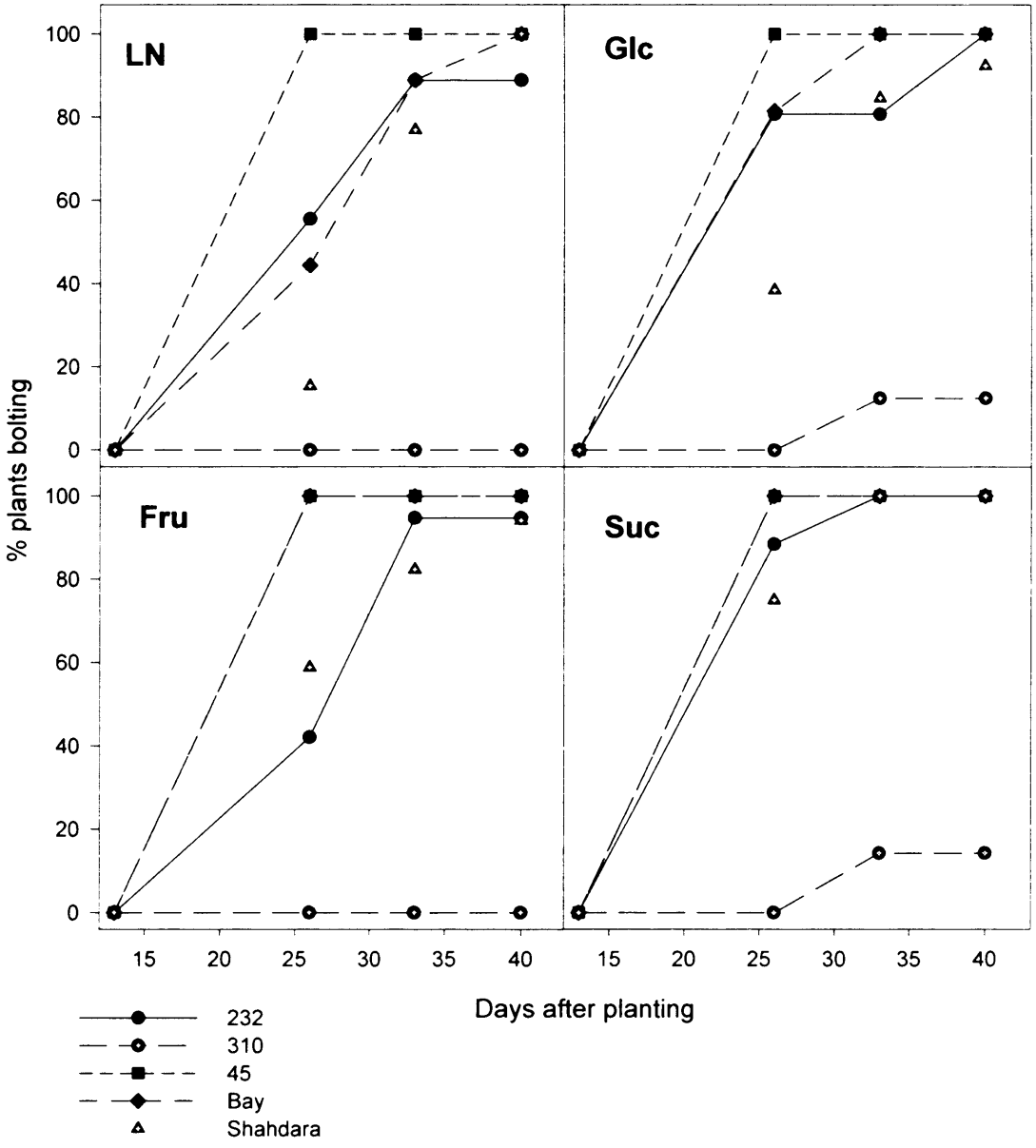


Figure 3.4. Bolting development of recombinant inbred lines and their parental accessions in response to treatment with low nitrogen and different sugars.

Bolting was denoted by the first appearance of the immature inflorescence and data are based on the percentage of the total number of plants bolting per RIL. A minimum number of 25 plants per RIL were observed.

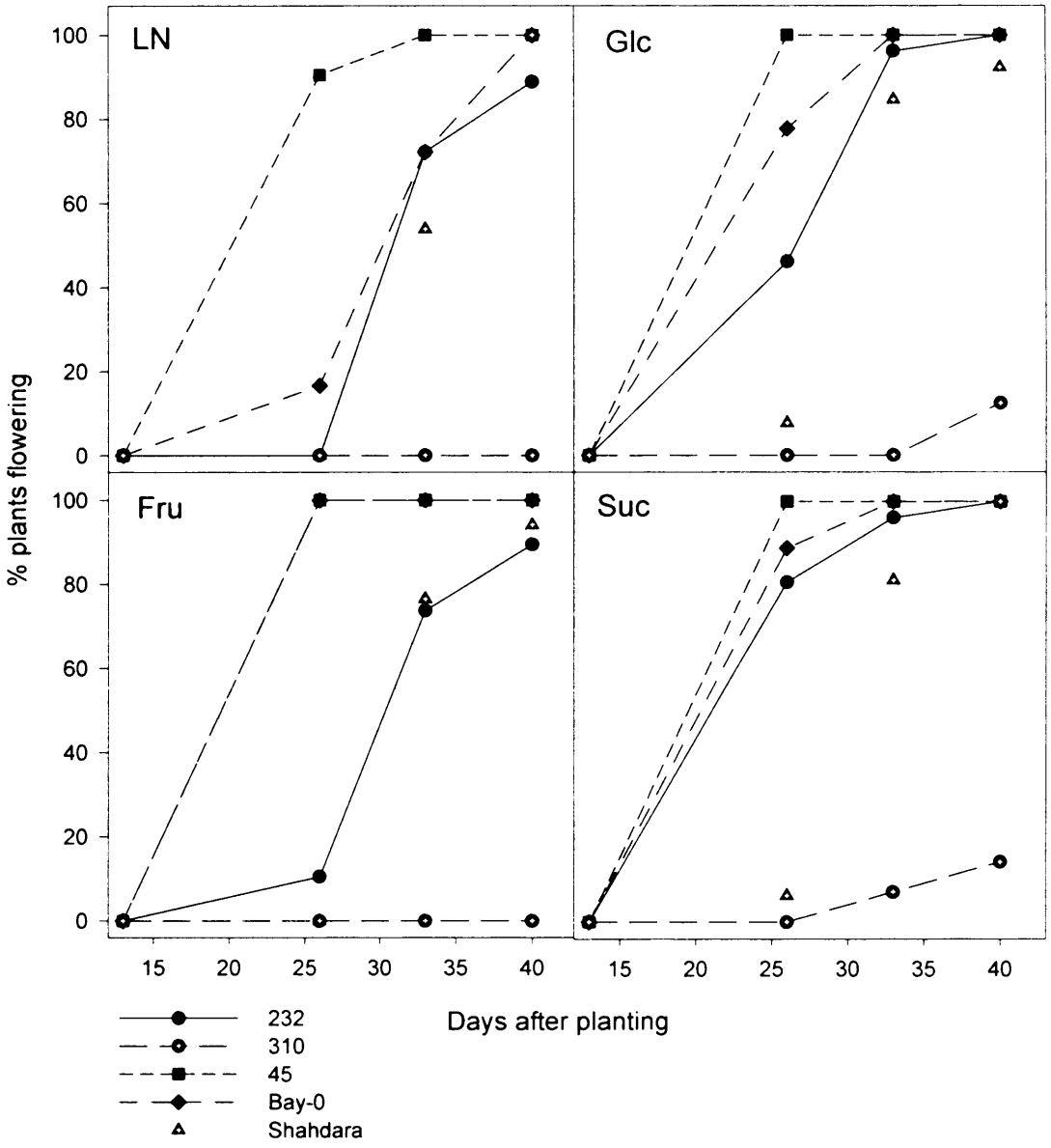


Figure 3.5. Flowering development of recombinant inbred lines and their parental accessions in response to treatment with low nitrogen and different sugars.

Flowering was denoted by first visual appearance of the flower petals, data are based on the percentage of the total number of plants flowering per RIL. A minimum number of 25 plants per RIL were observed.

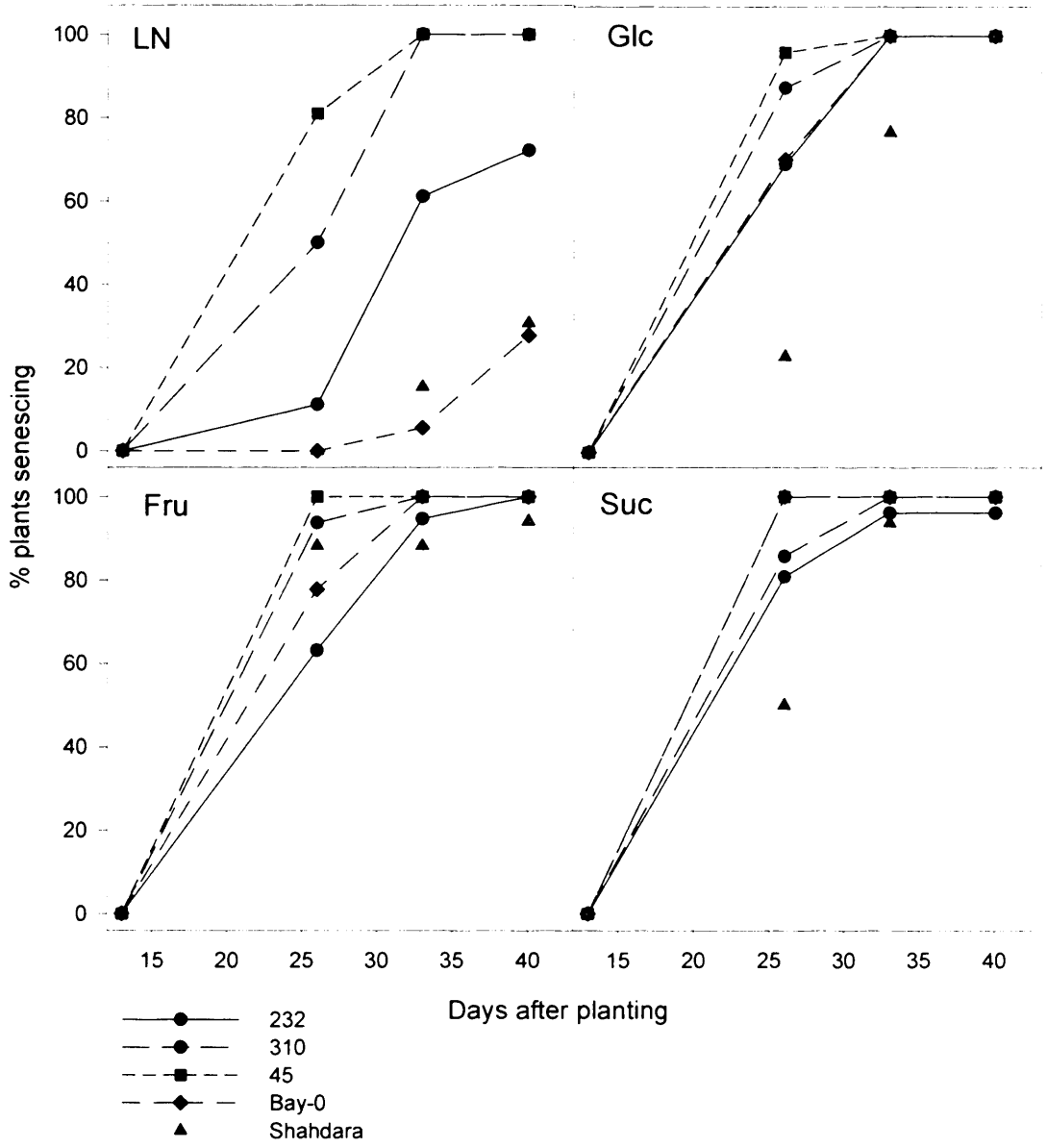


Figure 3.6. Senescence development of recombinant inbred lines and their parental accessions in response to treatment with low nitrogen and different sugars.

Senescence was denoted by visual yellowing and data is based on the percentage of the total number of plants senescing per RIL. A minimum number of 25 plants per RIL were observed.

3.3 Discussion

3.3.1. The sugar regulated senescence is not caused by osmotic stress

Maximum photosynthetic efficiency (F_v/F_m) is an effective measure of senescence as the values represent the state of the chloroplast which is progressively dismantled as senescence progresses. Plants treated with glucose showed an accelerated senescence phenotype (lower F_v/F_m) compared to treatments with mannitol or sorbitol. The glucose senescence response is therefore caused by glucose signalling and/or metabolism and not merely due to its osmotic effect.

Arroyo et al. (2003) showed that *ABI4* (*abscisic acid insensitive 4*) transcription could be induced by mannitol but in notably lower quantities than by glucose. Furthermore, when glucose and mannitol were compared in their ability to regulate the expression of *CTR1* (*constitutive triple response 1*) it was observed that both glucose and mannitol caused initial repression but whereas the glucose repression was transient and recoverable, mannitol resulted in a permanent decline. The conclusion of these observations was that glucose signalling dominates osmotic signalling in the regulation of the *CTR1* gene (Arroyo et al. 2003). These finding are evidential that osmotic stress and glucose signalling instigate different signalling pathways but the study by Arroyo et al. (2003) much higher (7%) concentrations of mannitol and sorbitiol were used. In this study the osmolyte and glucose concentrations used (111 mM) are too low to cause osmotic stress, as proved by the lack of response to mannitol and sorbitol and the similarity in response to the osmolytes and low nitrogen (LN), in the RILs.

3.3.2. The Bay-0 x Shahdara population display a range of responses in treatment with nitrogen and glucose.

The RILs treated with high nitrogen (HN) high nitrogen + 2% glucose (HNG) and low nitrogen (LN) retained a high F_v/F_m throughout the experiment. The most striking response to treatment was seen in treatment with low nitrogen and glucose (LNG) in which most lines started to decline rapidly after day 28. Line 310 and Shahdara, however, displayed a high F_v/F_m throughout the trial whereas RILs 45 and Bay-0 declined rapidly after only DAP 20.

The accelerated decline in maximum photosynthetic efficiency (F_v/F_m) in treatment with low nitrogen plus 2% glucose (LNG) as opposed low nitrogen (LN) is probably the result of an imbalance in the sugar to nitrogen ratio being sensed within the leaf. A study by Wingler et al. (2004) observed a decline in F_v/F_m, the quantum efficiency of energy capture by photosystem II and the quantum efficiency of photosystem II electron transport when *Arabidopsis* (Col-0) plants were grown in LNG conditions compared to other combinations of nitrogen and glucose. These findings strongly suggest that sugar regulated senescence is dependent on nitrogen status and the carbon: nitrogen ratio acts as the initial signal (Masclaux et al. 2000; Pourtau et al. 2004).

A study by Paul and Driscoll (1997), observed the effect of nitrogen deprivation and shading (to limit carbohydrate accumulation) in tobacco. The authors observed that in nitrogen deprived, but unshaded plants more carbon accumulated within the leaves

and concluded that this occurred because carbon was not being utilized for nitrogen assimilation. As a consequence of this accumulation of leaf sugars, photosynthesis was repressed but this resulted in the carbon:nitrogen balance being restored (Paul and Driscoll, 1997).

The Bay-0 x Shahdara population displayed a wide range of responses to treatment and exhibited phenotypes outside of their parental ranges. This observation was supported by a study of flowering time conducted by Loudet et al. (2002), in which highly significant differences ($P = <0.001$) between the RILs was observed in response to treatment with long or short daylengths, despite, in the aforementioned study, there being very little phenotypic difference between the parental accessions (Loudet et al. 2002). Additionally, QTL studies into the control of yellowing and anthocyanin accumulation in this RIL family, resulted in the identification of fifteen new loci and, again, observed transgressive segregation which were greater than the parental values (Diaz et al. 2005). This latter study measured yellowing both visually and with image processing and found that digital image processing uncovered five new loci, compared to four with visual analysis (Diaz et al. 2005). From my own observations and supporting literature it was thus concluded that the Bay 0 x Shahdara RIL population and maximum photosynthetic efficiency (F_v/F_m) would be suitable tools for the further investigation of senescence mechanisms.

The observation of the five recombinant inbred lines and their parental accessions revealed that RILs 83 and 272 were phenotypically similar to RIL 232 (Figure 3.2) it was therefore decided that forthcoming experiments would omit these two lines and

characterisation would focus on RILs 232, 310, 45 and their parental accessions, Bay-0 and Shahdara.

3.3.3. Sugars need to be metabolised for signalling to occur

Having confirmed that senescence induction was not caused by an osmotic effect, the impact of different metabolisable sugars was compared. In this experiment RILs from the Bay-0 x Shahdara population were used. Treating the RILs with the different sugars revealed little difference between the treatments. This is possibly because the sucrose was rapidly hydrolysed into glucose and fructose. For this reason a direct signalling function of the sucrose molecule has been hard to establish (Smeekens, 2000). A sucrose specific signalling mechanism has been proposed by Rook et al. (1998), however, in their observations of a leucine zipper gene in Arabidopsis, *ATB2*. The expression of this gene is controlled by light through the gene products of *DET1* and *COP1*. The group observed that the expression of *ATB2* was specifically repressed by sucrose and that glucose and fructose were ineffective in mimicking this response (Rook et al. 1998). It is therefore possible that sucrose has a specific signalling function but the similarity in response to treatment in the experiments presented herein suggest that the sucrose was probably hydrolysed and then signalling was instigated by glucose and/or fructose.

The role of glucose as a potent signalling molecule has been reported in a number of studies (Ehness et al. 1997; Arroyo et al. 2003; Masclaux-Daubresse et al. 2007; Pourtau et al. 2006). There is strong evidence to suggest that hexokinase signalling may be responsible for the observed carbon:nitrogen regulation of senescence. This enzyme has been widely implicated as the initial sugar sensor in plants and yeast

(Jang and Sheen, 1997). High levels of sugar within leaf tissues repress photosynthesis and a cause a decrease in the Calvin cycle enzymes (Smeekens, 2000). This response was observed when cells of *Chenopodium rubrum* were treated with glucose but the non-phosphorylable analogs 6-deoxy-glucose and 3-O-methyl-glucose were ineffectual. In Arabidopsis, senescence is not induced by treatment with 3-0-methyl-glucose (A.Wingler, personal communication), showing that sugar phosphorylation is required for the senescence response. A study by Pourtau et al. (2006) demonstrated that the hexokinase mutants and *gin2-1* exhibited a delayed senescence phenotype when grown on glucose containing medium compared to wild type plants. The authors thus concluded that hexokinase is required for glucose regulation of senescence (Pourtau et al. 2006). Based upon this assumption any metabolisable sugar could be used in the growth media to test the senescence response of the RILs but as glucose is the most readily phosphorylated sugar, and a number of studies have already demonstrated its signalling capabilities, glucose was chosen as the sugar to be tested in subsequent experiments.

Chapter 4

Characterisation of the Bay-0 x Shahdara recombinant inbred line population

4.1 Introduction

Recombinant inbred lines (RILs) offer tremendous potential for genetic studies in plant science. The homozygous genome of several hundred closely related individuals is advantageous over exclusively using mutant plants because it allows the dissection of complex polygenic traits such as nutrient use efficiency (Loudet et al. 2003), flowering time (Werner et al. 2005) and senescence (Diaz et al. 2006; Luquez et al. 2006). These traits are the product of multiple loci as opposed to single, unique mutations, therefore a more comprehensive analysis of the genome is required to elucidate the underlying mechanisms (Loudet et al. 2002).

The Bay-0 x Shahdara population created by Loudet et al. (2001) is a relatively new RIL population. The two wild-type parent plants Bay-0 and Shahdara originate from fallow land in Bayreuth, Germany and the Pamiro-Alay mountains, Tadjikistan, respectively (Loudet et al. 2002a). Recent studies have demonstrated that Central-Asian Arabidopsis ecotypes represent genetically isolated populations compared to the widely hybridised European varieties (Loudet et al. 2002a; Sharbel et al. 2000; Erschadi et al. 2000; Breyne et al. 1999; Innan et al. 1997) offering a tool with which to identify and probe interesting and unusual agronomic traits.

The study of the hybrid (homogenised) progeny of the Bay-0 x Shahdara population has already uncovered the genetic basis for a number of complex traits such as flowering (Loudet, et al. 2000), yellowing and anthocyanin accumulation (Diaz et al. 2006) and nitrogen use efficiency (Loudet et al. 2003). For this reason the potential benefits of using this population for my studies were apparent.

Based on studies conducted by Diaz et al. (2005) recombinant inbred lines, including the two parental accessions, were selected for characterisation studies. The purpose of this was to observe the relationship between particular phenotypic features and the senescence phenotype e.g. sugar content and maximum photosynthetic efficiency (F_v/F_m). It was also important to establish which of these features would be most suitable to act as the quantifiable characteristic for subsequent QTL analysis. The selected lines were named/numbered as follows: Bay-0 (female parent), Shahdara (male parent), 232, 310 and 45. In the initial trials two extra RILs, 272 and 83 were included but as they showed similar phenotypes to 232 and 45, respectively, they were not included in subsequent experiments. The parameters chosen were maximum photosynthetic efficiency (F_v/F_m), chlorophyll, leaf sugar contents and the development of bolting, flowering and senescence. The expression of ten genes in response to treatment was observed using RT-PCR. The genes were chosen based on micro-array data (accession number: E-MEXP-387; www.ebi.ac.uk/arrayexpress) taken from the selected RILs and represented the transcripts that were regulated by treatment with 2% glucose (Masclaux-Daubresse et al. 2007; Appendix 4.1).

The chosen parameters are all established measures of senescence. Maximum photosynthetic efficiency F_v/F_m has been used as a measure of senescence in a number of studies (Pourtau et al. 2006; Araya et al. 2006; Wingler et al. 2004; Oh et al. 2000). It can be determined non-invasively allowing changes to be observed over the course of plant development which allows the time point at which senescence is initiated in different plants to be identified. Previous observations have shown that visible signs of senescence, such as yellowing, occur concurrently with a drop in F_v/F_m (Diaz et al. 2005; Wingler et al. 2004). Chlorophyll content is therefore a measurement complimentary to F_v/F_m as chlorophyll is progressively degraded as senescence progresses (see main introduction). Both chlorophyll a and chlorophyll b contents were measured and this allowed a greater insight into both breakdown and the relationship of both chlorophylls to F_v/F_m to be elucidated. A QTL study of senescence in an indica/japonica hybrid in rice used chlorophyll content as one of the quantified traits measured (Farouk et al. 2005). The authors uncovered that a locus involved in chlorophyll content at flowering time colocalised with a previously discovered locus involved in senescence (Toojinda et al. 2003) and that a QTL on chromosome IV (*Oryza sativa* L.) colocalised with a locus for retention of greenness of the flag leaf previously identified by Jiang et al. (2004) (Farouk et al. 2005).

A micro-array study conducted by Pourtau et al. (2006) investigated the effects of high nitrogen plus 2% glucose, low nitrogen and low nitrogen plus 2% glucose on the *Arabidopsis* accession Ws-2. Results of this study identified a number of genes that were strongly altered in their expression by glucose. The authors then carried out RT-PCR experiments to further support their findings. Of particular notability was a MYB transcription factor, *PAP2* was strongly up-regulated, and more strikingly a 900

fold increase in the expression of Senescence Associated Gene12 (*SAG12)* was observed in treatment with low nitrogen plus 2% glucose (LNG). Conversely this treatment had a negative effect on the expression of the chloroplast glutamine synthetase gene, *GS2* (Pourtau et al. 2006).

The three genes cited above that were shown to be greatly affected by LNG were used in this study to observe whether the same pattern would be maintained in the Bay-0 x Shahdara population. A CATMA-array study (Masclaux-Daubresse et al. 2007; Appendix 4.1) had also identified six other genes that were significantly altered in their expression. These genes were also tested.

4.2 Results

4.2.1. Maximum photosynthetic efficiency (F_v/F_m) of the Bay-0 x Shahdara population in response to treatment

Five recombinant inbred lines (RILs) and their parental accessions were selected from the 420 line collection based on previous studies (Masclaux-Daubresse et al. 2007; Diaz et al. 2005; Loudet et al. 2004.). A more rapid decline in F_v/F_m was observed in plant treated with LN plus 2% glucose (LNG) compared to low nitrogen only (LN) (Figure 4.1). RIL 310 retained a high F_v/F_m in both treatments whereas RILs Bay-0, 272 and 45 declined much more rapidly in treatment with glucose after DAP 25. By DAP 35 all lines were senescing, except, notably, 310 which maintained an F_v/F_m >0.75 until after DAP 40. This result is very unusual as accelerated decline in response to LNG has been observed in all other Arabidopsis accessions previously tested including Ws-2 (Pourtau et al. 2006), Col-0 (Wingler et al. 2004; Pourtau et al. 2004), L*er*-0, L*er*-2, Cvi, Nd-1, Col-5, Kas1-1 and Kas2-1 (Levey and Wingler, 2005).

The very obvious effect of LNG on senescence compared to either HN or HNG treatment (see Chapter 3) supported previous evidence that senescence is regulated by disequilibria between leaf sugar content and nitrogen. Only LN and LNG treatments were used henceforth.

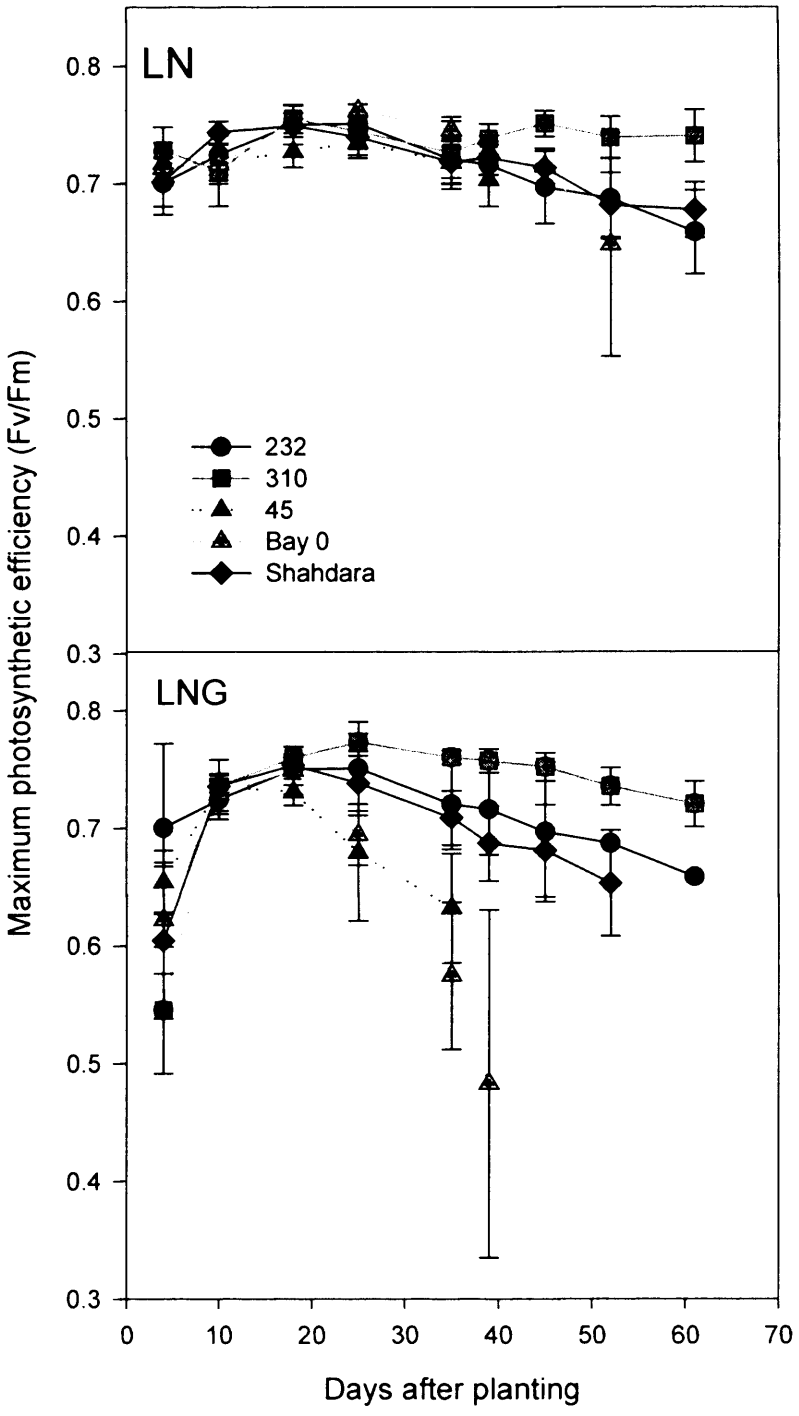


Figure 4.1. Maximum photosynthetic efficiency (F_v/F_m) of recombinant inbred lines (RILs) and their parental accessions in response to treatments with nitrogen and glucose.

Recombinant inbred lines 232, 310, 45, Bay-0 and Shahdara were grown in two treatments: low nitrogen (LN) and low nitrogen + 2% glucose (LNG). Data is based on a minimum of 25 plants per RIL and error bars denote standard deviation.

4.2.2. Physical appearance of recombinant inbred lines (RILs) 35 days after planting

The physical appearance of the three RIL and the two parental accessions displayed variable phenotypes and a high degree of transgression (Figure 4.2 and 4.3). In all lines senescence was accelerated when plants were grown on media containing 2% glucose (LNG). The exception to this was RIL 310 in which F_v/F_m was only very slightly reduced in LNG. At the last time point (DAP 61) in LNG this RIL had an F_v/F_m of 0.72 compared to 0.74 in LN. It can be seen from Figure 4.2 that in LNG treatment only the very oldest leaves of RIL 310 (visible in the bottom right hand corner of the picture) were showing signs of senescence whereas in treatment with LN only, senescence was much more advanced and affecting all leaves.

From the flowering data (Figure 4.4) it can be seen that flowering was also severely delayed in RIL 310, indeed, no bolting or flowering was observed in plants in treatment LN and <40% flowered in LNG treatment. This lack of reproductive development was accompanied by continued production of new leaves at the top of the rosette when all other lines had stopped producing new leaves. This pattern of development in RIL 310 was particularly pronounced in LNG treatment. Also affected in its flowering phenotype by treatment with low nitrogen and glucose (LNG) was RIL 232. Flowering was delayed by LNG treatment, compared to LN, by an average of 5 days. The male parent, Shahdara, also exhibited late flowering but this was found in both treatments. This result is surprising because Shahdara has been cited by a number of studies as an early flowering accession (Luquez et al. 2006;

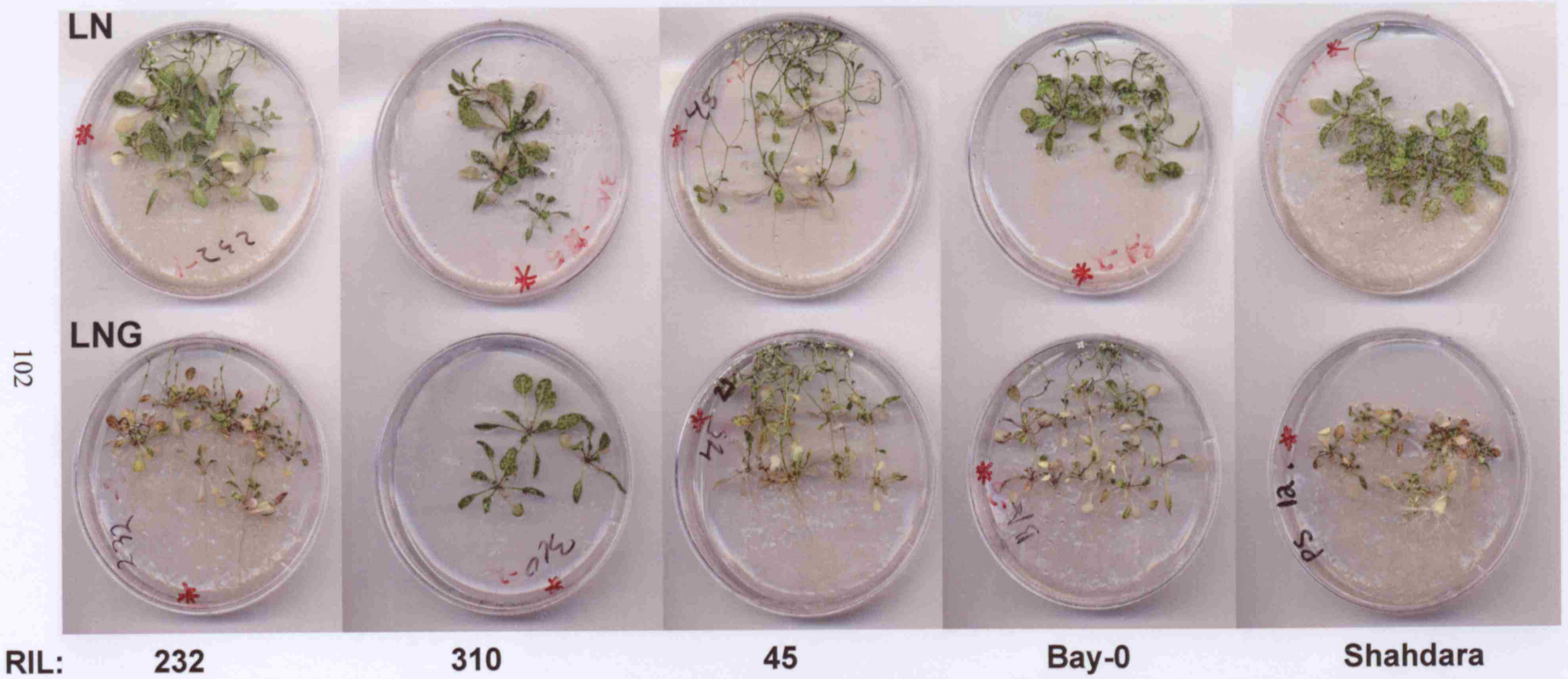


Figure 4.2 Recombinant inbred lines (RILs) and their parental accessions in response to treatment with low nitrogen (LN) and low nitrogen plus 2% glucose (LNG)

All pictures were taken 30 days after planting

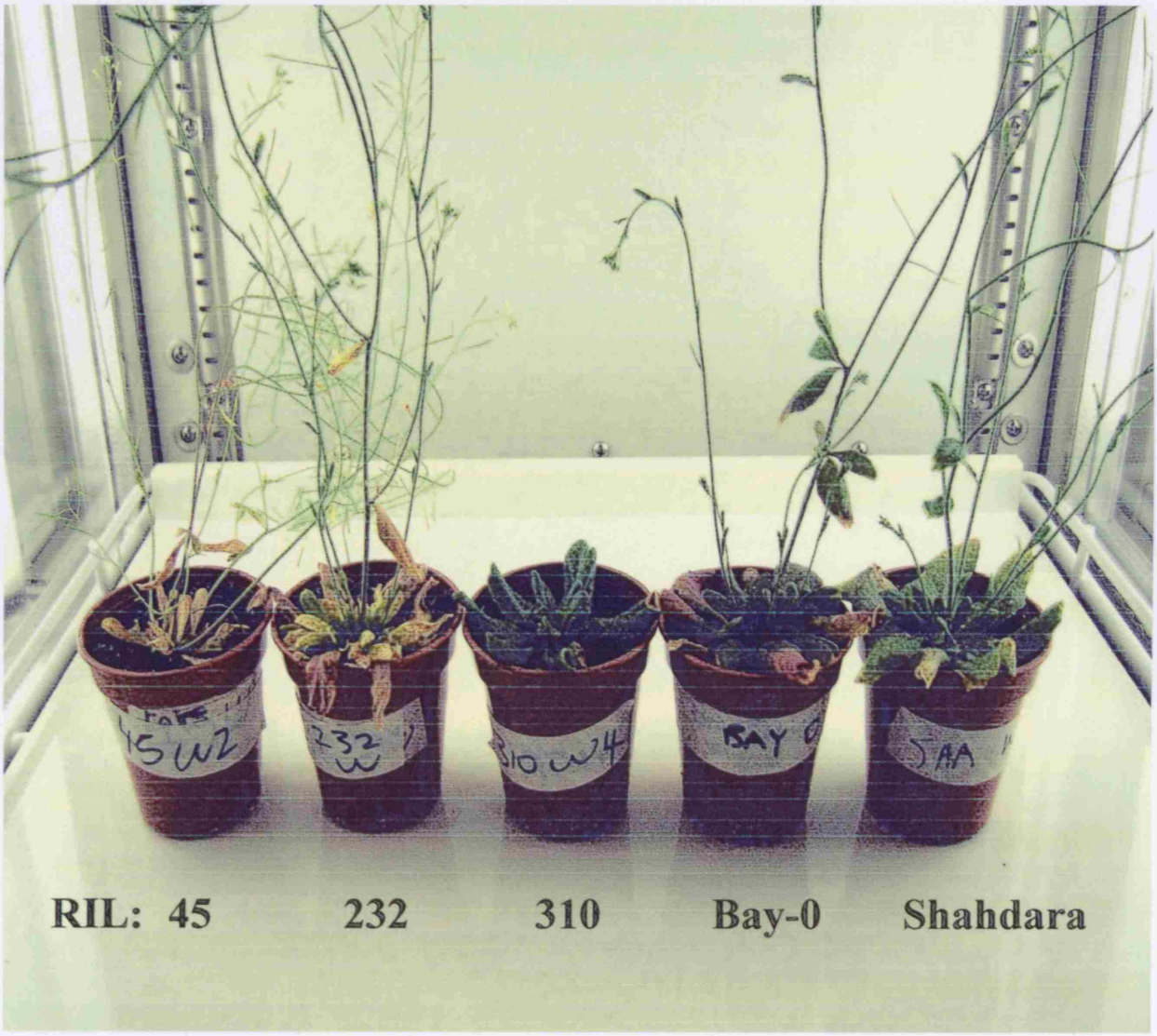


Figure 4.3 Recombinant inbred lines (RILs) and their parental accessions grown in compost*.

Pictures taken at DAP 42

*Pictures courtesy of Thushiyanthi Sivagnanam

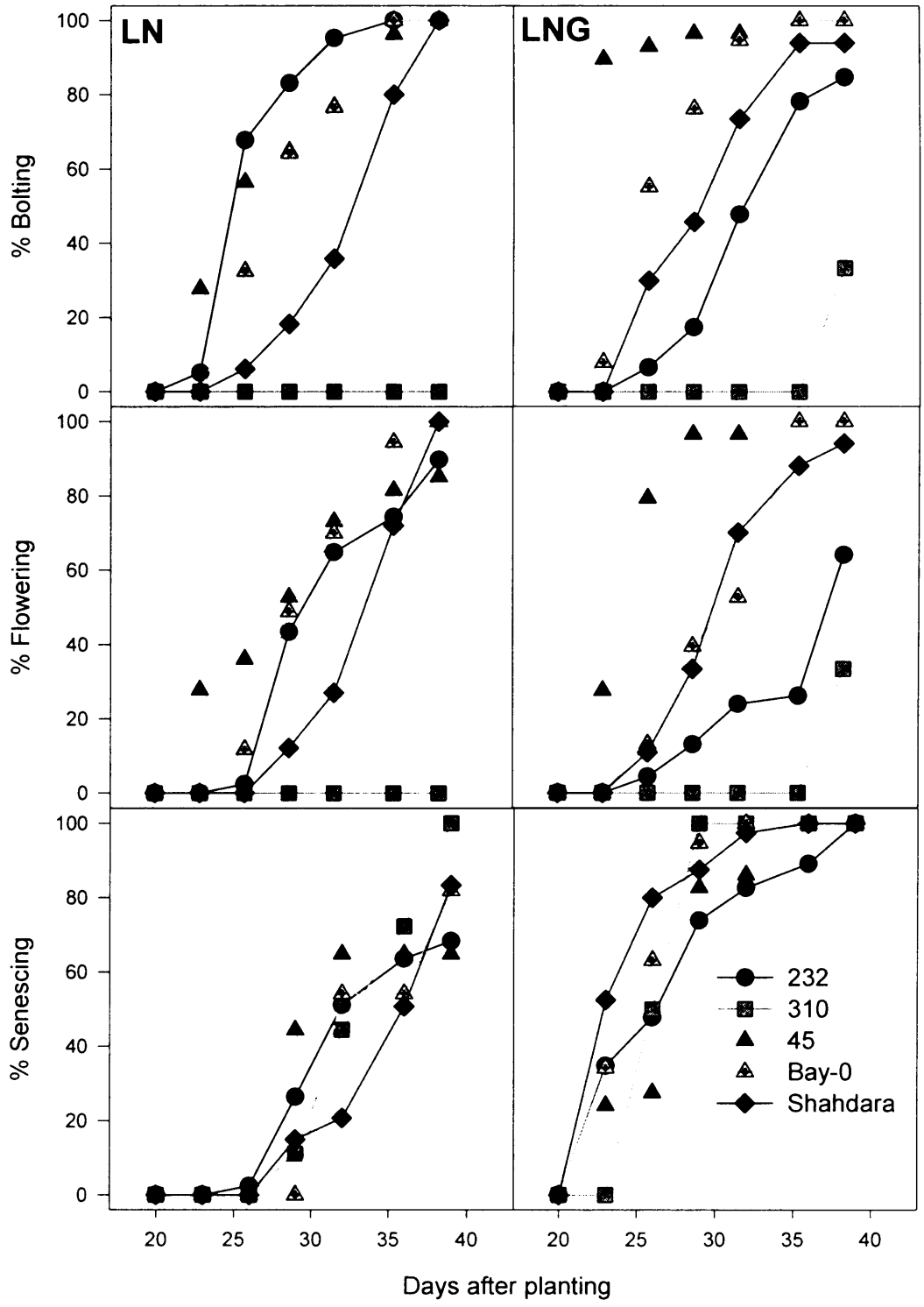


Figure 4.4. Development of selected recombinant inbred lines (RILs) and their parental accessions in response to treatment with low nitrogen (LN) or low nitrogen plus 2% glucose (LNG). Data is based on a minimum of 25 plants per RIL. Bolting was denoted by the appearance of the immature florescence, flowering by the appearance of flower petals and senescence by visual yellowing. Data was calculated as a percentage of the total number of plants per RIL.

Werner at al. 2005) but from these results it is implied that this is a nitrogen-status dependent characteristic. RIL 45 and Bay-0 switched to reproductive growth more quickly in treatments with glucose; Bay-0 started bolting an average of 3 days before its non-glucose treated counterpart and RIL 45 only took <30 days in treatment with LNG to achieve 100% flowering compared to 36 days in LN treatment.

4.2.3. Chlorophyll contents of RILs grown in low nitrogen plus 2% glucose

The chlorophyll contents of the selected recombinant inbred lines (RILs) varied in response to LNG treatment (Figure 4.4). RIL 310 and 232 had the highest total amounts of chlorophyll and RIL 310 also had the highest ratio of chlorophyll a:b. The high amount and high ratio of chlorophyll found in RIL 310 is unsurprising due to the senescence phenotype of this line (Figure 4.1) which was characterised by the continuous production of new leaves in the upper rosette. The RIL with the lowest total chlorophyll (Figure 4.5A) was line 45 with only 0.18 μg/mg FW, this line exhibits an accelerated senescence phenotype when treated with LNG (Figure 4.1 and 4.2).

Figure 4.5B shows the relationship between chlorophyll and F_v/F_m. No strong correlation between total chlorophyll and F_v/F_m was observed, for example Shahdara had the lowest total chlorophyll content but still had the second highest F_v/F_m. A relationship was observed, however, between chlorophyll a:b ratio and F_v/F_m. In this case it can be seen that Shahdara had the second highest ratio of chlorophyll a: b, after 310.

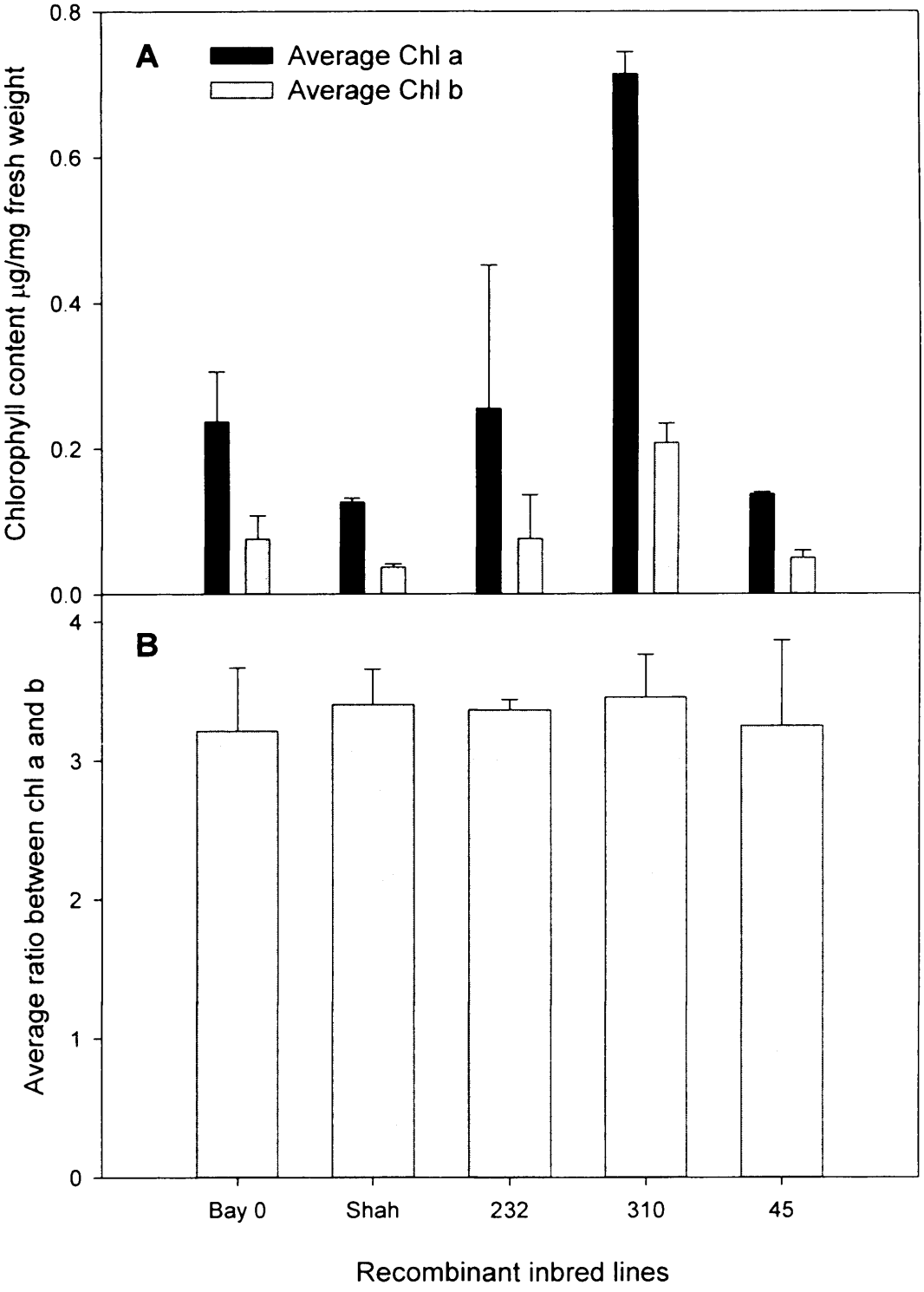


Figure 4.5. A: Amount of chlorophyll a and b (µg/mg fresh weight) and B: Average ratio between chlorophyll a and b in recombinant inbred lines (RILs) and their parental accessions.
All plants were grown in low nitrogen + 2% glucose and data harvested 35 days after planting. Data are based on the average values of 3 plants per RIL. Error bars show standard deviation.

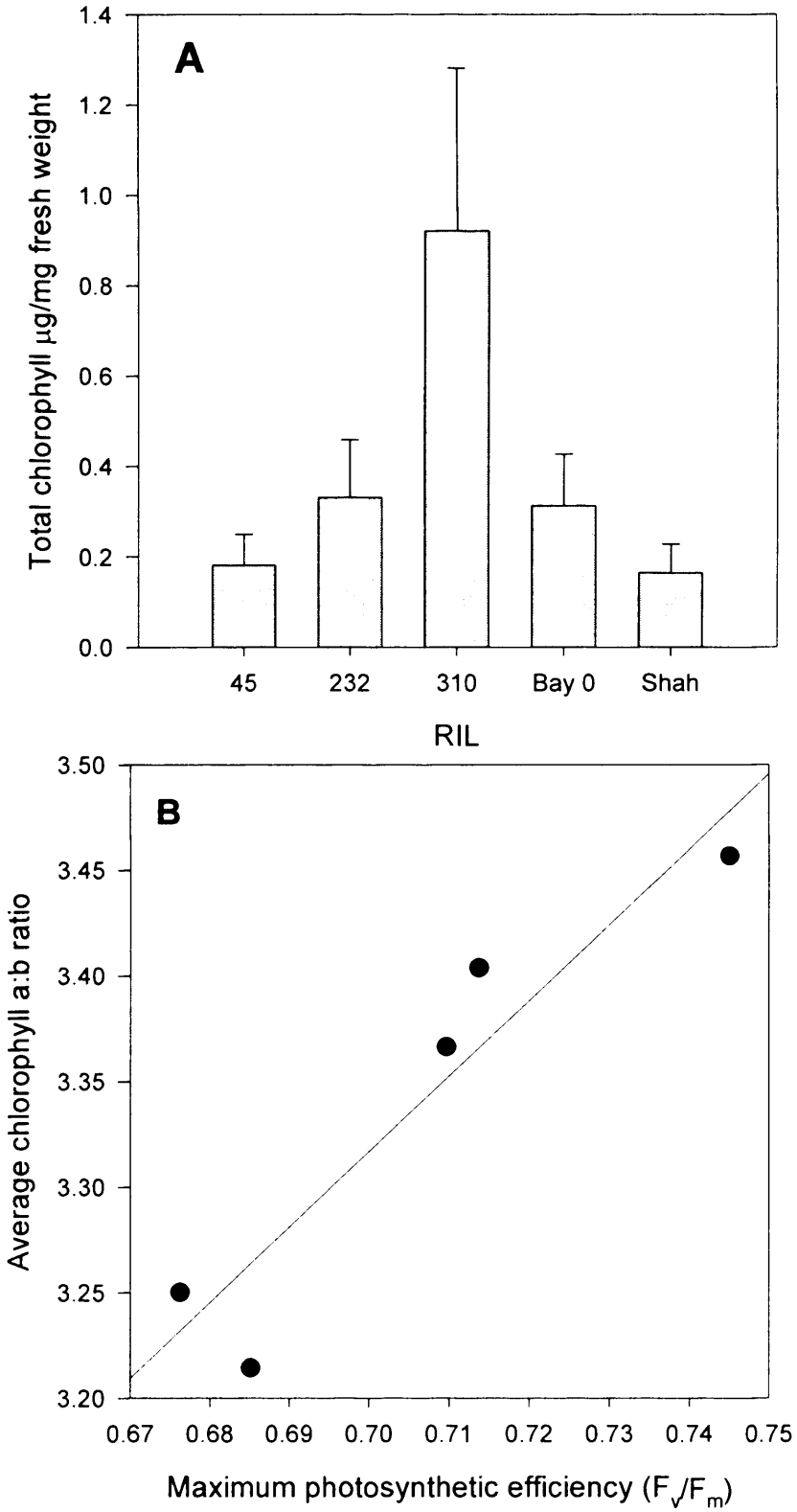


Figure 4.6. A: Total chlorophyll and B: Relationship between chlorophyll a:b ratio and maximum photosynthetic efficiency (F_v/F_m) Data for chlorophyll is based upon the average value of three plants per RIL. Data for the F_v/F_m is based on a minimum of 25 plants per RIL. All data from plants were grown in LNG and harvested at DAP 35. Error bars denote standard deviation.

and had the second highest F_v/F_m value at this time point. Similarly lines 45 and Bay-0 that had the lowest ratios (3.21 and 3.25, respectively) also had the lowest maximum photosynthetic efficiency values (0.67 and 0.68). A correlation equation revealed an R value of 0.94 (Appendix 4.2) showing a positive correlation between the chlorophyll a:b ratio and F_v/F_m. No correlation between total chlorophyll and F_v/F_m was observed.

4.2.4. Leaf sugar contents

Leaf sugar contents were up to five times as high in plants grown in low nitrogen plus 2% glucose (LNG) compared to low nitrogen only (LN) (Figure 4.7). In all lines an increase was observed between DAP 30-40 in LNG, except the glucose content of 310. Conversely, an increase in the sucrose content of all lines was observed in LN but differences were slight for glucose and sucrose in all lines in this treatment. Despite this the greatest increase in leaf sugar content was seen in the sucrose content of RIL 310 in LN which increased by over 65% between DAP 30 and 40.

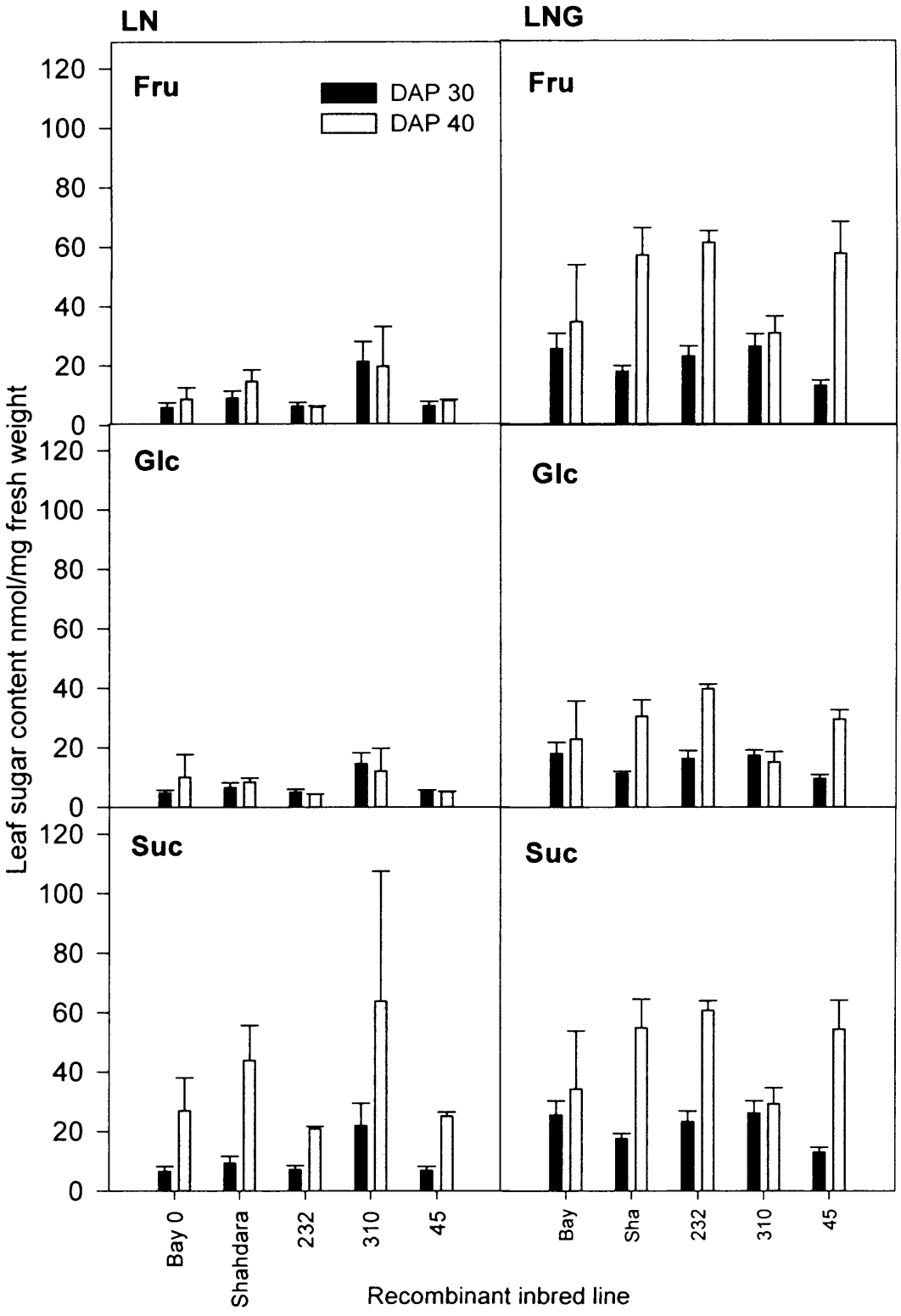


Figure 4.7. Leaf sugar contents of recombinant inbred lines treated with either low nitrogen (LN) or low nitrogen plus 2% glucose (LNG). Data was collected at two time points 30 (black bars) and 40 (grey bars) days after planting. Data is based on three samples per RIL. Error bars denote standard deviation.

4.2.5 Gene expression in response to treatment

For full list of genes and respective AGI codes see Materials and Methods, Chapter 2.

SAG12 (At5g45890) (Figure 4.8) was up-regulated strongly in LNG treatment in RILs Bay-0, Shahdara, 232 and 45 (Figure 4.8). In these RILs no expression was observed in LN. This result was expected as previous studies had shown that this gene was strongly expressed in glucose treated Ws-2 plants but much less in treatment with LN only (Pourtau et al. 2006). In RIL 310, however, the opposite was observed and expression was only observed in LN treatment. This result is interesting because 310 is the only RIL in which complete rosette senescence is delayed by the application of glucose (Figures 4.1 and 4.2). Expression was most strongly observed in LNG Bay-0. RIL 232 showed the least expression in both treatments.

PAP2 followed almost exactly the same expression patterns as SAG12; it was up-regulated by glucose in all RILs except 310, which was also the only line to show expression in LN (Figure 4.8). The strongest induction of *PAP2* by glucose was found in the lines with the strongest senescence phenotypes, 45 and Bay-0.

A *jacalin* gene, (At2g39330) was up-regulated by treatment with LNG (Figure 4.8). This was most pronounced in Shahdara and also RIL 310. At this time point these were the two least senescent lines (Figure 4.1).

Cor15b (At2g42530) was up-regulated by glucose in all treatments (Figure 4.8). The biggest difference between LN and LNG was found in RIL 310 in which hardly any

expression was observed in treatment with LN. RIL 45 showed very low expression in both treatments. RIL 232 showed the highest expression in both LN and LNG, followed in this trend by Shahdara. It must be noted, however, that the expression of the control gene, *ACT2*, was slightly higher in RIL 232 and this may have resulted in higher expression being observed for the other genes tested.

GER1 (At1g72610) was down regulated by glucose in all lines except 232 which did not show any effect, and RIL 310 which showed induction in the absence of glucose.

ASN2 (At5g65010) was down regulated by treatment with low nitrogen plus 2% glucose in all lines except 310 in which a slightly stronger signal was detected in LNG (Figure 4.8). Expression was still strong in RILs Shahdara and 232 when treated with LNG but was low in the two most senescent lines, 45 and Bay-0.

Expression of *GS2* (At5g35630) showed down regulation by glucose (LNG) in all lines except 310, where a weak signal was detected in LNG only. For RIL 232 no signal was detected in LNG treated plants even though the strongest expression of all lines in either treatment was observed in LN.

ACT2 (At3g18780) was used as the control gene as it was similarly expressed in all RILs in both treatments.

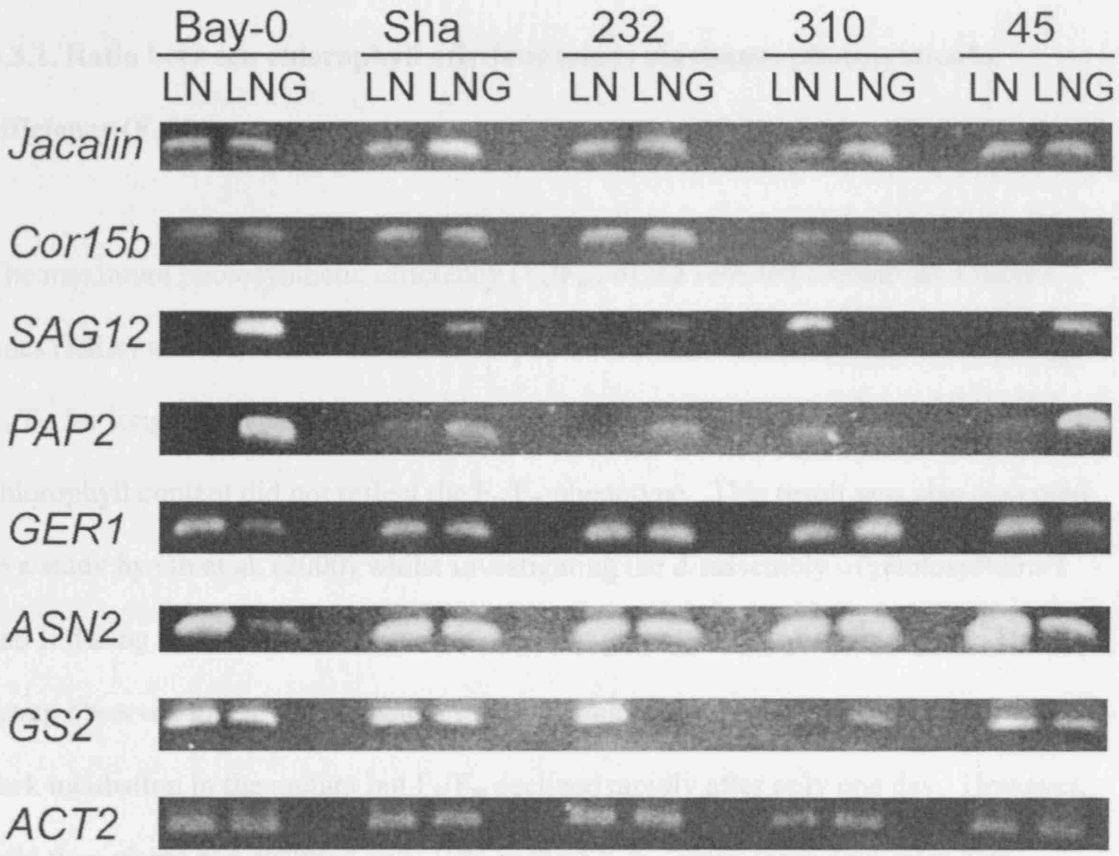


Figure 4.8. Reverse transcription polymerase chain reaction (RT-PCR) results of the expression of eight genes. Plants were treated with low nitrogen (LN, left bands) or low nitrogen + 2% glucose (LNG, right bands). Band sizes were confirmed with use of a 100bp ladder. Recombinant inbred lines were harvested for above data 30 days after planting.

4.3 Discussion

4.3.1. Ratio between chlorophyll a:b determines maximum photosynthetic efficiency (F_v/F_m)

The maximum photosynthetic efficiency (F_v/F_m) of the selected recombinant inbred lines (RILs) was reflective of their chlorophyll a:b ratio. Lines that maintained a high F_v/F_m for longer generally had a high ratio of chlorophyll a:b and vice versa. Total chlorophyll content did not reflect the F_v/F_m phenotype. This result was also observed in a study by Oh et al. (2000) whilst investigating the disassembly of photosystems I and II during dark induced senescence of a stay-green mutant of Arabidopsis. The group observed that a "significant amount" of chlorophyll was retained five days after dark incubation in the mutant but F_v/F_m declined rapidly after only one day. However, wild type plants had declined only 10% in their F_v/F_m value three days after dark incubation but chlorophyll content had dropped by 70%. This rapid drop in total content was due to rapid degradation of chlorphyll b, which in turn increased the chlorophyll a:b ratio. The group therefore concluded that although the total number of antenna complexes had decreased in the early stages of senescence the function of the remainder was unaffected. Consequently, the wild type plants that retained a high ratio of chlorophyll also retained a F_v/F_m throughout the five day trial compared to the mutant with higher overall chlorophyll (Oh et al. 2000). It is probable therefore, that a late senescing line such as Shahdara, that had a low overall chlorophyll content compared to an early senescing (decline in F_v/F_m) line like Bay-0, was the result of the number, integrity and efficiency of the remaining photosystems as opposed to

simply the quantity of light absorbing pigments available. Furthermore, retaining high quantities of chlorophyll whilst the electron transport chain was being degraded could have accelerated senescence as a result of photo-oxidative damage caused by dis-equilibrium in the capture and dissipation of energy (Wingler et al. 2004).

A higher ratio of chlorophyll a: b was observed in the late senescing RILs 310 and Shahdara. During chlorophyll degradation chlorophyll b is first converted to chlorophyll a because the key enzyme in the degradation of chlorophyll is pheophorbide oxygenase and this enzyme only accepts pheide a, which is produced from chlorophyll a (Scheumann et al. 1998). Therefore, when the amount of each chlorophyll was measured some of the chlorophyll a would have originated from chlorophyll b. The combination of chlorophyll a and b acts to stabilize the chlorophyll a/b binding protein by forming a folded structure (Ito et al. 1996). Therefore the conversion of chlorophyll b to a probably exposes the binding proteins to the action of chlorophyllases during senescence. As it appears from the results presented herein that the ratio, rather than quantity, of chlorophyll is the important factor in maintaining photosynthetic efficiency, the late senescing lines were probably still able to regulate the ratio between chlorophyll a and b at this later time point. Evidence for this is suggested by Ito et al. (1996) who observed that during adaptation to high light conditions chlorophyll a was converted to chlorophyll b to maintain the formation of the core complexes of the photosystems. This demonstrates that chlorophyll ratios are regulated to respond to changing conditions and so it is plausible that the less senescent RILs were able to maintain the most efficient ratios and consequently photosynthetic integrity.

4.3.2. Sugars increase in LNG treated plants during senescence

The content of sugars within the leaves of plants grown in low nitrogen and 2% glucose (LNG) was up to five times as high as in plants grown in low nitrogen only. The average increase between LN and LNG was 2.0 fold higher at day 30 and 2.2 fold higher at day 40 in LNG treated plants. In a study of the effect of carbohydrate accumulation on photosynthesis in bean (*Phaseolus vulgaris*) plants treated with 20 mM of sucrose to their roots were found to have a 1.5-3 fold increase in leaf sugar content which is consistent with the findings presented here (Araya et al. 2006).

On LN, sucrose, but not glucose and fructose contents increased between day 30 and 40. In plants grown in the presence of glucose, with the exception of 310, all the sugars increased between day 30 and day 40. The first time point measurement at DAP 30 marks the early stage of senescence and the latter time point, DAP 40, late senescence. The accumulation of sugars as senescence progresses has been reported previously (Wingler et al. 2004; 2005; 2006a; Pourtau et al. 2003). An explanation for this is offered by Pourtau et al. (2004) who observed that sugars only accumulate in low nitrogen, not high nitrogen treated plants, and this could result from a lower utilization of sugars for the production of amino acids and proteins (Pourtau et al. 2004).

For all RILs grown in LNG the amount of glucose within their leaves was lower than fructose or sucrose this is probably because glucose is rapidly converted to provide energy for cellular processes. When high levels of glucose are available it has been

hypothesised, based upon studies with yeast, that genes involved in the utilization of sucrose may be down regulated (Lalonde et al. 1999) resulting in an accumulation as witnessed in this study.

RIL 310 is different in that flowering is severely delayed but vegetative growth is characterised by the progressive death of old leaves followed by a new wave of leaf production at the top of the rosette. Therefore, the lower leaf sugar contents on LNG at DAP 40 in RIL 310 were possibly the result of measurements being taken from younger leaves compared to the other RILs, i.e at DAP30 mature leaves were measured but by DAP 40 the second wave of new leaves, with low sugar contents were measured. The change in leaf sugar content depending on leaf age has been reported previously. For example, in mature tobacco leaves, as were probably measured in 310 at DAP 30, are characterised by having a high carbohydrate content whereas young leaves have a low carbohydrate content (Masclaux et al. 2000). Furthermore, in *Phaseolus vulgaris* the young sink leaves of plants treated with 20 mM sucrose still contained lower amounts of carbohydrate then the old source leaves of non-sugar treated plants (Araya et al. 2006).

A second explanation for the lack of sugar accumulation during senescence in RIL 310 is offered by Diaz et al. (2005) who measured the amino acid contents of all the RILs used in this study apart from RIL 45. The authors observed that the content of a number of amino acids, including asparagine, leucine and isoleucine was unusually high in RIL 310 compared to the other lines tested (Diaz et al. 2005). Furthermore GABA (γ-aminobutyric acid), a product of the glutamate decarboxylase reaction was found to accumulate in 310. A possible role of GABA in senescing tissues is to act as

a transient nitrogen storage compound (Diaz et al. 2005; Masclaux et al. 2000) and this hypothesis is supported by its concurrent accumulation around the nitrogen-rich amino acids in RIL 310 (Diaz et al. 2005). Different contents of amino acids in RIL 310 were also found by Masclaux et al. (2006). It is therefore probable that sugars are being constantly utilized in this line to produce amino acids resulting in lower leaf storage.

4.3.3. Selected gene expression is regulated by sugars and nitrogen

The instigation of senescence by sugars is supported by the reverse transcriptase polymerase chain reaction (RT-PCR) results. The results clearly show that *SAG12*, a senescence specific gene, was strongly up-regulated by glucose in all the RILs, except 310 in which a higher F_v/F_m is observed when the plants are treated with glucose at DAP 30 than without. *SAG12* cannot be induced by wounding or stress in young leaves (Weaver et al. 1998) and so is used as an indicator of senescence (Wingler et al. 2006). The role that *SAG12* plays in regulating senescence is unclear. It encodes a cysteine protease which suggests that it may be involved in protein breakdown but the very late expression of this gene during senescence, continuing after most protein degradation has occurred, suggests it has a different role to play. Furthermore, *SAG12* expression is regulated by salicyclic acid (Morris et al. 2000) and mutant plants defective in the salicylic acid signalling pathway show reduced expression of *SAG12* but normal chlorophyll and protein degredation. Morris et al. (2000) thus concluded that *SAG12* may cause the transition from senescence to necrosis.

That *SAG12* was strongly up regulated in all lines that showed an accelerated senescence phenotype in response to low nitrogen + 2% glucose (LNG) is compelling evidence that glucose induces natural (non stress induced) senescence and, if Morris et al. (2000) are correct in their postulations, glucose continues to regulate senescence until death occurs. The expression of *SAG12* in *Brassica napus*, was found to be increased in response to low nitrogen conditions. Specifically, the authors noticed that expression matched the sink-source transition; expression was highest in mature leaves containing low nitrogen and lowest in young leaves (Gombert et al. 2006). Although the authors did not measure leaf sugar contents of their plants, carbohydrate content has been shown to increase with leaf age in Arabidopsis (Wingler et al. 2005; 2006), tobacco (Mascleaux et al. 2000) and bean (Araya et al. 2006). It is therefore probable that the decrease in nitrogen in this study was actually coupled with an increase in sugar content. This could have resulted in the observed increased in expression of *SAG12*, as found in my own studies and that of Pourtau et al. (2006).

A study by Pourtau et al. (2006) identified that *SAG12* was induced up to nine hundred fold in *Arabidopsis* (Ws-2) plants treated with 2% glucose compared to control plants, which is complementary to the data presented herein. The induction of *SAG12* in the absence of glucose in RIL 310 confirms that this line has unusual senescence characteristics.

Anthocyanins have been reported to play a protective role in senescence by acting as scavengers for reactive oxygen species during the breakdown of the chloroplast during senescence (Diaz et al. 2005). It would therefore be expected that the more senescent and vulnerable to oxidative damage the RILs were would be reflected in the

expression of *PAP2*. The expression patterns of *PAP2* (production of anthocyanin pigment 2), a MYB transcription factor, were almost identical to *SAG12* supporting this prediction. The most senescent lines when grown in LNG medium, 45 and Bay-0, showed the highest expression whereas the less senescent 232 and Shahdara showed less expression as was mirrored in expression of *SAG12*. Similar to *SAG12*, *PAP2* was induced on glucose-free medium in 310, confirming the senescence phenotype. It has been reported previously that this gene can be induced by sugars (Pourtau et al, 2006) which is supported by this study. The results presented herein also support a postulation expressed by Pourtau et al (2006) that *PAP2* demonstrates an antagonistic relationship between sugars and nitrogen. The repression of *PAP2* by nitrogen was reported in a study by Scheible et al. (2004) and was supported in a study by Pourtau et al. (2006), who observed that this gene was expressed in treatments with LNG but not HNG. That this gene is strongly up-regulated in treatment with LNG compared to LN (as found in this study, also) supports the study by Pourtau et al. (2006) in the claim that *PAP2* is a regulator of senescence by responding to changes in the sugar:nitrogen status of senescing leaves.

Three of the genes that were observed, *GER1* (germin gene), *ASN2* (asparagine synthetase) and *GS2* (glutamine synthetase) were down regulated by glucose treatment in Bay-0, Shahdara, 45 and 232 but not in 310. A possible explanation for this result has been offered through further study with *GS2*.

GS2 encodes a chloroplast glutamine synthetase gene which is involved in ammonia assimilation during photorespiration. This result has been previously observed in an Affymetrix GeneChip analysis of Arabidopsis Ws-2 plants (Pourtau et al. 2006) and

also a CATMA array (Masclaux-Daubresse et al. 2007; Appendix 4.1). Further investigation revealed that *GS2* is highly sensitive to nitrogen status and that glucose feeding can induce nitrogen starvation. The Affymetrix GeneChip analysis of Arabidopsis Ws-2 plants revealed that when plants were grown in high nitrogen conditions the expression of *GS2* remained low but stable, whereas at low nitrogen supply expression was repressed in comparison (Pourtau et al. 2006). This is probably because senescence is regulated by the ratio of sugar to nitrogen within the leaf (Masclaux-Daubresse et al. 2005; Diaz et al. 2005) and a degree of competition between pathways exists. This may result in a reduction of glutamine synthetase, as was observed in the expression patterns of *GS2* in response to treatment with low nitrogen plus 2% glucose.

A gene involved in cold tolerance, *COR15b*, was up-regulated in all lines but particularly strongly in 310, when plants were grown in LNG growth conditions compared to LN. A correlation between leaf sugar contents and freezing tolerance has been previously observed (Wanner and Junttila, 1999). It is possible that a feed forward loop may exist in the relationship between sugars and cold tolerance (Masclaux-Daubresse et al. 2007). It is known that cold treatment results in the accumulation of sugars and proline (Thomashow, 1999) and therefore it is possible that, likewise, sugars can induce genes involved in cold tolerance such as *COR15b*. The strong induction of *COR15b* and of other cold response genes in RIL 310 could indicate that the cold acclimation pathway is particularly induced by glucose in this line (Masclaux-Daubresse et al. 2007). Cold acclimation can result in decreased sugar sensitivity (Strand et al. 2003), and cold-acclimated plants do not show sugar-induced

senescence (Masclaux-Daubresse et al. 2007). This could explain why RIL 310 does not respond to glucose treatment in the same way as the other lines.

The up-regulation of the *jacalin* gene in LNG-treated plants, again particularly in 310, is indicative of a stress response. This gene is activated by jasmonic acid a hormone that has been implicated in response to various stresses such as a build up of ROS (Overmyer et al. 2003) and drought stress (He et al. 2002). The accelerated senescence phenotype observed in response to treatment with LNG in all lines tested, except 310, has been proved in this study not to be the result of osmotic stress (see Chapter 3) from the growth media. However, whether an external osmotic stress is imposed by the elevated sugar contents is a possibility and, if so, may have resulted in the expression of the *jacalin* gene. A second possibility is that the expression of *jacalin* is involved in defence against free radicals during senescence which may build up during chlorophyll detoxification (Hörtensteiner et al. 1998). The specific role of this gene in senescence has not been elucidated by this study. Levels of jasmonic acid and its precursor OPDA were measured but no differences were observed either between the RILs or between treatment with LN or LNG (see Appendix 4.4).

Expression of *SAG12* was only observed in RIL 310 when the plants were grown in LN conditions. Also the expression patterns of genes *PAP2*, *ASN2*, *GER1* and *GS2* showed the opposite expression patterns to that of all the other RILs. RIL 310 is also unusual because it has a higher F_v/F_m at DAP 30 with glucose compared to without. At DAP 30 the sugar contents of this plant were the highest on a fresh weight basis on LNG medium of all RILs so clearly 310 does not have a deficiency in storing sugars. However, by DAP 40 this RIL had the lowest leaf sugar content and was much more

photosynthetically active than the other RILs. This RIL continues to produce new leaves at the top of the rosette and it has been observed previously that young leaves of tobacco (Masclaux et al. 2000) and bean (Araya et al. 2006) contain less sugar than old leaves. Overall, RIL 310 showed an unusual senescence phenotype and unusual response to sugar treatment. The genetic basis of this was analysed in the following chapter.

A further observation of RIL 310 was lack of seed viability (data not shown). Many seeds would fail to germinate or become stunted shortly after cotyledon opening. Nooden and Penney (2001) offer an explanation for this by their observation that the production of additional young leaves increased infertility. It therefore appears that the reduced sugar accumulation at a late stage disrupted the trade-off between investment in reproduction and photosynthetic capacity (Levey and Wingler, 2005).

Chapter 5

Quantitative trait loci analysis using recombinant inbred lines

5.1 Introduction

Quantitative trait loci (QTL) analysis has been used to establish the genetic basis behind a diverse range of traits in wide variety of plant species. QTL for senescence have been mapped in tomato (Paterson et al. 1988), wheat (Verma et al. 2004), rice (Jiang et al. 2004) and Arabidopsis (Luquez et al. 2006; Diaz et al. 2006). QTL analysis marks the first stage in the genetic dissection of traits of interest. The complete sequencing of the Arabidopsis genome makes subsequent, molecular analyses of uncovered loci possible and thus the identification of specific genes of agronomic importance (Loudet et al. 2002).

The use of QTL analysis relies on a detailed genetic linkage map of the organism. Principally, a linkage map indicates the relative genetic distance and position of markers along the chromosomes (Collard et al. 2005). Mapping relies on the recombination (crossing over) frequency of markers during meiosis. Markers that are closely positioned will segregate less frequently than those that are more spatially separated. The frequency with which particular markers recombine in the segregating progeny can be used to calculate the order and relative distance between markers along the chromosomes (Collard et al. 2005).

The Bay 0 x Shahdara population has been extensively mapped using DNA based markers at microsatellite loci showing polymorphic simple sequence repeats: ((AT)*n* or (AG)*n*, where *n*> 12), every 10-15cM along the five chromosomes (Loudet et al. 2002). Polymorphic DNA based markers have the advantage over morphological or biochemical markers because they can reveal the underlying genetic differences between plants of the same species (Collard et al. 2005). As only robust markers were used to deduce the genotype of the F8 recombinant inbred population used in this study, the precision of the genetic map is very high.

The underlying principle of QTL analysis is that genes of interest are positioned next to, or between, genetic markers. Consequently, these genes will recurrently occur along with these particular markers and will be inherited along with the marker in resulting progeny. A quantitative phenotypic feature, in this study the maximum photosynthetic efficiency (F_v/F_m), is chosen as a measure of the trait of interest, such as senescence. The analysis is performed statistically based on the mean values of the quantifiable trait (F_v/F_m) at a given time point. The values of the trait are statistically compared to the incidence of the different markers in the genotype. Each marker is assigned a value based upon whether it has an additive effect on the trait of interest (higher F_v/F_m) or a reductive effect (lower F_v/F_m). A logarithm of odds (LOD) score is then computed to determine whether the incidence of a particular marker with an F_v/F_m value is the result of chance or reflects a linkage between the marker and the gene of interest. The significance threshold of a LOD score depends on the replication and population size. A LOD score of 3 is indicative that linkage is 1000 times more likely than no linkage (Collard et al. 2005). For the results presented herein A LOD score of 2.4 was considered to be significant. The minimum number of

individuals required to create a linkage map is 50 (Young, 1994) and so in a large RIL population like Bay 0 x Shahdara the risk that a correlation between a specific marker and a specific F_v/F_m value is the result of chance is greatly reduced. In this study QTL Cartographer was used for the statistical probability analyses as this software has proved effective in previous studies conducted on the Bay-0 x Shahdara population (Loudet et al. 2002; Diaz et al. 2005).

The accuracy of QTL mapping has gained support since genes located by QTL analyses have been successfully tagged or cloned and were found to be very close to the initial loci. El-Din El Assal et al. (2001) conducted a study into flowering time in Arabidopsis. The group generated near isogenic lines and used map-based cloning to uncover that an identified QTL accounting for 20-55% of flowering time variation was a novel allele of *CRY2*, a gene that promotes flowering under long day conditions (El-Din El Assal et al. 2001; Alonso-Blanco et al. 1998). The location of this allele was found to be within 0.8-1.6 cM from the initially discovered QTL peak with an average distance of only 0.1 cM (El-Din El Assal et al. 2001; Price 2006). Other studies conducted on the protein content of wheat (Joppa et al. 1997), flowering time in soybean (Watanabe, 2004; Yamanaka et al. 2005) and transpiration efficiency in Arabidopsis (Masle et al. 2005) all proved that the genes of interest mapped to <2 cM away from the QTL peaks, thus supporting QTL analysis as an accurate method of identifying genes of interest.

After locating a chromosomal region of interest by QTL analyses an established protocol is to then nominate candidate genes. Candidate genes have been defined as: "genes with molecular polymorphisms genetically linked to major loci or QTLs, or genes with molecular polymorphisms statistically associated with variation of the trait

being studied" (Pflieger et al. 2001). The extensive bioinformatic databases available for the Arabidopsis genome, e.g. TAIR, allows positional candidate genes to be nominated based on location alone. A criticism of QTL analysis, however, is that the identified regions of interest may span several mega bases making positional candidate gene nomination inaccurate (Dupuis and Siegmund, 1999) but many studies have now proved the worth of the candidate gene approach as their postulations have been later proved by genetic studies using cloning or transformation.

A detailed knowledge of the biochemical and metabolic processes involved in governing a trait of interest is essential in candidate gene nomination. For example, Beavis et al. (1991) conducted a QTL study on maize to locate genes involved in determining plant height and located a region on chromosome 9. It was previously known that two genes, *dwarf3* and *phytochrome B*, had effects on plant height (Fujoika et al. 1988; Boylan and Quail 1991). The biological functions of these genes were analysed and found to encode proteins involved in gibberellin biosynthesis and light detection, respectively, which were found to co-segregate with the QTL (Beavis et al. 1991). A subsequent study was then carried out to identify which of the two nominated genes was actually responsible for the phenotype. Near isogenic lines were generated and demonstrated that *dwarf3* showed a polymorphism between the lines strongly suggesting that this was the gene of interest (Touzet et al. 1995).

Similar studies involving QTL analyses and candidate gene nomination have been carried out for a diverse range of traits including water stress (Pelleschi et al. 1999), carbon metabolism (Causse et al. 1995) and protein and starch content (Goldman et al. 1993). These studies have proved that QTL analysis, the use of near isogenic lines and candidate gene nomination have provided the essential groundwork for

subsequent genetic analyses that have ultimately concluded with the identification of the specific genes involved in governing agronomically important traits.

Previous studies had determined that loci involved in plant longevity that were identified in low nitrogen were different to those uncovered in high nitrogen conditions (Luquez et al. 2006). Furthermore, studies by Diaz et al. (2006) had uncovered loci involved in senescence under nitrogen limitation in the Bay-0 x Shahdara population. As sugars are important regulators of senescence, the aim of the work presented in this chapter was to identify loci involved in the sugar response during senescence. In addition to the sugar treatment, for example, the main difference between the work presented here and the QTL analysis by Diaz et al. (2006) is that Diaz et al. (2006) analysed senescence in the first six leaves, whereas here whole rosette senescence was analysed by fluorescence imaging. In contrast to senescence of the first leaves, whole rosette senescence is linked to flowering (Levey and Wingler, 2005). It was therefore expected that in addition to some of the QTL mapped by Diaz et al (2006) further QTL would be detected. These QTL could, for example, underlie the regulation of floral transition or sugar sensitivity.

5.2 Results

5.2.1. Frequency distribution of F_v/F_m phenotypes

The histogram of data taken at 36 DAP showed relatively normal distribution (Figure 5.1). Most of the RILs (82%) clustered within values of 0.62 - 0.74 but 63% of RILs exhibited a more extreme phenotype, in either direction, than their parental accessions; Bay-0 = 0.68 and Shahdara = 0.71. Many of the RILs had lower values than the parental accessions but only 16% had higher values than Shahdara. RIL 310 displayed the most extreme phenotype as characterised in Chapter 4, with the highest F_v/F_m values. Some lines showed very low F_v/F_m values, either indicating early senescence or a general stress response.

The broad sense heritability of the glucose-regulated senescence was calculated from a selection of 24 RILs by an analysis of variance model (ANOVA) (Table 5.1). Heritabilities ranging from 46 – 72% (DAP 32 and 43, respectively) were observed, indicating that approximately 50% of the total variation could be attributed to genotype.

5.2.2. Identification of loci controlling glucose induced senescence

The application of exogenous glucose and growth in low nitrogen conditions accelerates senescence in Arabidopsis. The Bay-0 x Shahdara population was grown under these conditions and the maximum photosynthetic efficiency (F_v/F_m) measured

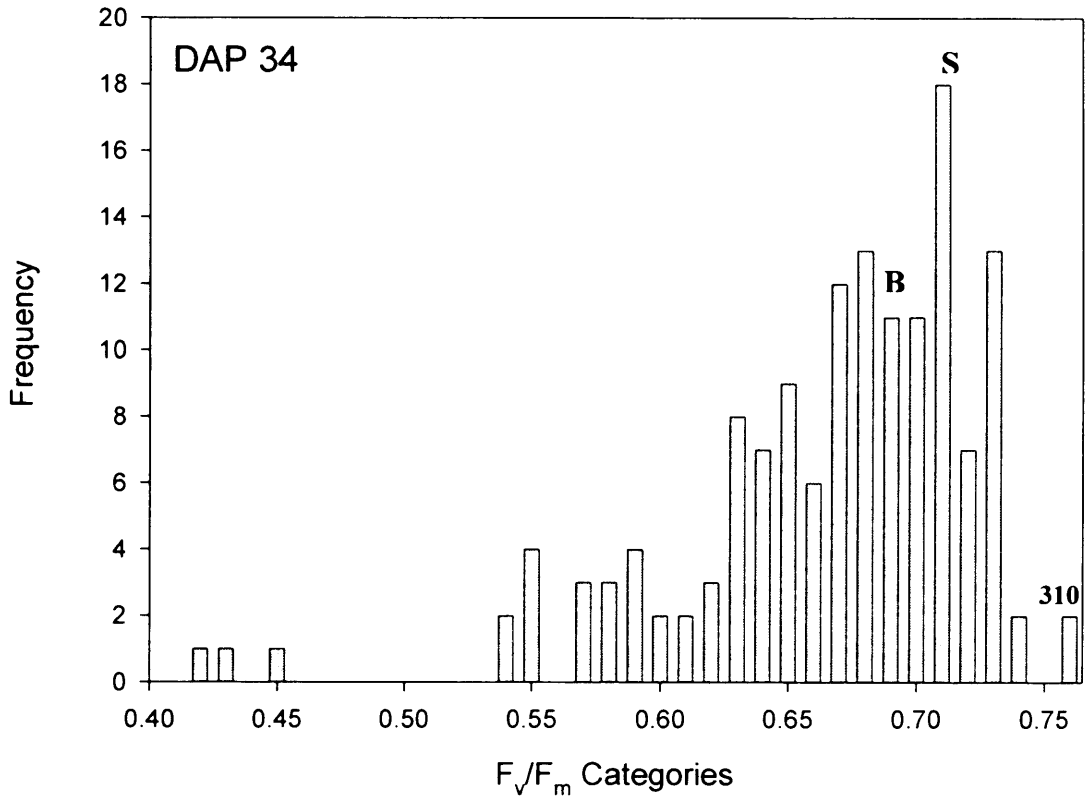


Figure 5.1. Cumulative frequency distribution of the maximum photosynthetic efficiency (F_v/F_m) 34 days after planting (DAP).

All plants were grown in low nitrogen plus 2% glucose (LNG) growth medium. Data is the average value of a minimum of 10 plants per recombinant inbred line (RIL). B: Bay-0 the female parental accession S: Shahdara the male parental accession. 310: RIL 310 exhibiting a transgressional phenotype.

Days after planting	% Heritability
14	0.62
24	0.53
32	0.66
48	0.78

Table 5.1 Broad sense heritability for glucose-induced senescence in recombinant inbred lines (RILs).

Heritability was calculated using an analysis of variance model (ANOVA). See materials and methods for details.

to act as the quantifiable characteristic for QTL analysis. The complete trial was replicated twice.

The log of odds (LOD) chromosome maps at DAP 30 and 40 (Figures 5.2 and 5.3) show significant peaks (> 2.4) on chromosomes II and IV. The peaks on Chromosome IV had LOD scores of 5.3 and 4.5 at DAP 30 and 40, respectively. Chromosome II had a peak > 4.2 at DAP 30. The loci on Chromosome II lie between 6-19 cM and on Chromosome IV between 0.01-12.2 cM.

In total, including results from two independent experiments and several time points, eleven QTL were detected using analysis of variance and composite interval mapping. They were located on chromosomes II, III and IV. The R^2 values, indicating the percentage contribution to the trait, ranged from 8 -18.21 (Table 5.2). In all loci, except GLISEN43ii on chromosome III, the Bay-0 allele had a negative effect on maximum photosynthetic efficiency (F_v/F_m), resulting in accelerated senescence.

Four of the detected loci; GLISEN30ii, GLISEN43i, GLISEN43ii and GLISEN34i, co-localised with the Y3.2, Y3.3 and Y3.4 loci of which Y3.2 (GLISEN30.2 and GLISEN43.1) is involved in leaf yellowing (Diaz et al. 2006) and Y3.3 and Y3.4 (GLISEN43.2 and GLISEN34.1, respectively) are involved in leaf yellowing and nitrogen use efficiency (Diaz et al. 2006; Loudet et al. 2003) (Table 5.2). Additionally, the loci detected on Chromosome IV (except GLISEN29.1) co-localised with the SD1 locus (Figure 4.3) which is involved in the control of short day flowering (Loudet et al. 2002) and also RV 3.4 involved in anthocyanin accumulation (Diaz et al. 2005).

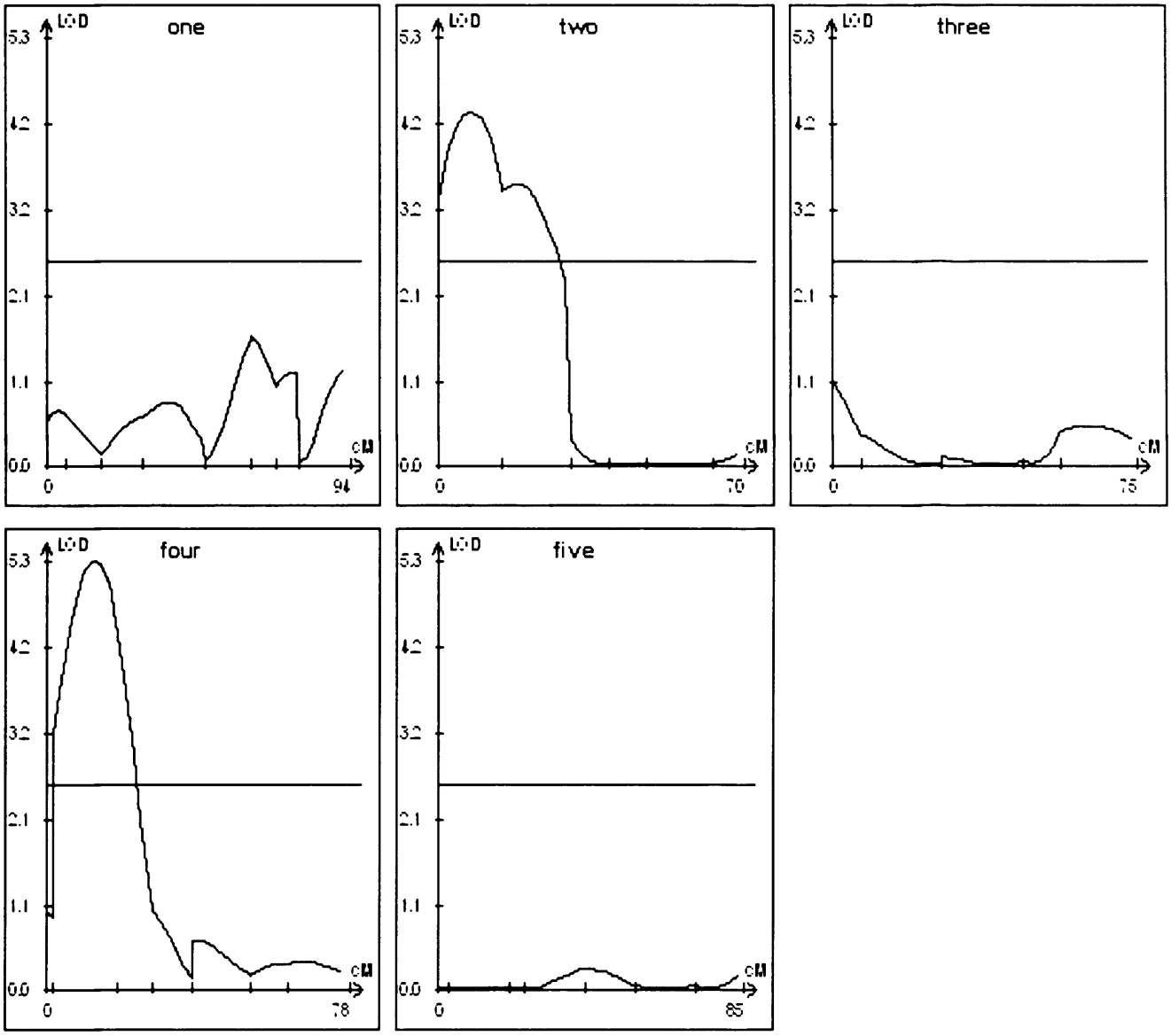


Figure 5.2. Quantitative trait loci likelihood of odds (LOD) chromosome maps for glucose induced senescence in low nitrogen plus 2% glucose 30 days after planting.

The horizontal axes correspond to the position of the locus in cM along each of the five chromosomes stated at the top centre of each graph. The vertical axes correspond to the LOD score; that is the likelihood that a particular value is the results of linkage rather than chance. A LOD score of 2.4 is significant. The data is based on the use of 180 recombinant inbred lines with a minimum of 20 plants per RIL. The quantifiable characteristic was maximum photosynthetic efficiency (F_v/F_m).

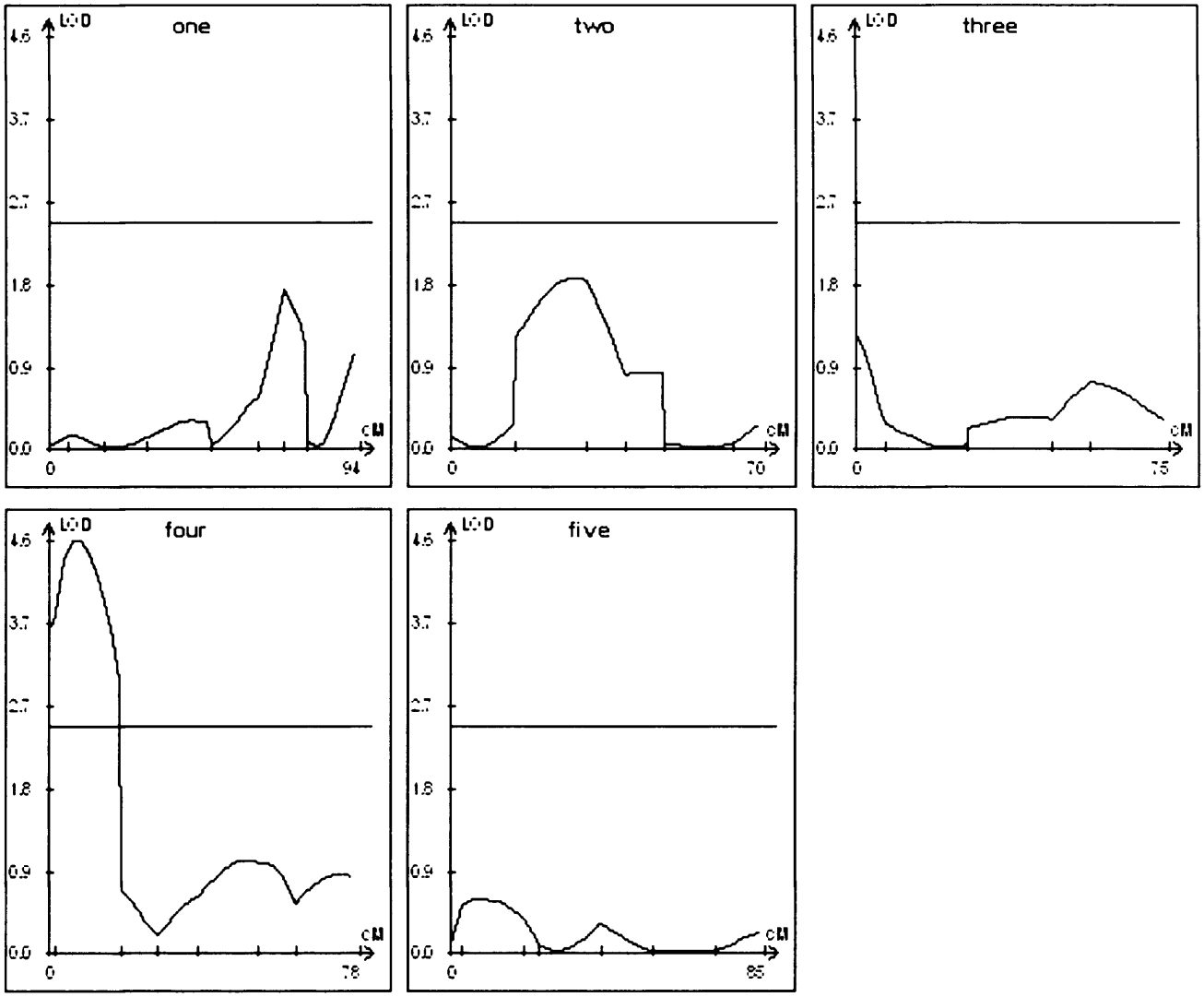


Figure 5.3. Quantitative trait loci likelihood of odds (LOD) chromosome maps for glucose induced senescence in low nitrogen plus 2% glucose 40 days after planting.

The horizontal axes correspond to the position of the locus in cM along each of the five chromosomes stated at the top centre of each graph. The vertical axes correspond to the LOD score; that is the likelihood that a particular value is the results of linkage rather than chance. A LOD score of 2.4 is significant. The data is based on the use of 180 recombinant inbred lines with a minimum of 15 plants per RIL. The quantifiable characteristic was maximum photosynthetic efficiency (F_v/F_m).

QTL	Chr-marker	Map position (cM)	LOD score	R^2	Allelic effect 2a	Corresponding QTL from Diaz et al. 2006
GLISEN 30i	Chrom2-MSAT2.5	6.51	3.14	10.99	-0.0077	-
GLISEN 30ii	Chrom2-MSAT2.38	18.46	2.79	9.55	-0.0072	Y3.2
GLISEN 43i	Chrom2-MSAT2.38	18.56	3.47	11.54	-0.0244	Y3.2
GLISEN 34i	Chrom3-MSAT3.21	53.33	3.8	11.72	-0.019	Y3.4
GLISEN 43ii	Chrom3-NGA172	1.61	3.34	9.55	+0.0223	Y3.3
GLISEN 29	Chrom4-MSAT4.39	0.01	3.7	8.68	-0.00868	-
GLISEN 30iii	Chrom4-MSAT4.8	12.02	3.68	12.9	-0.0084	-
GLISEN 34ii	Chrom4-MSAT4.8	8.82	5.34	15.97	-0.022	-
GLISEN 36i	Chrom4-MSAT4.8	3.22	3.6	11.42	-0.0282	-
GLISEN 40i	Chrom4-MSAT4.8	8.92	3.4	18.21	-0.0287	-
GLISEN 43iii	Chrom4-MSAT4.8	5.12	4.72	14.89	-0.0279	-

Table 5.2. Summary of QTL involved in glucose induced senescence.

QTL are listed as GLISEN (glucose induced senescence) followed by the day that they were detected. Chr-marker denotes the nearest micro-satellite marker to the locus and map position in centi Morgans is shown. The R^2 value indicates the total percentage allelic contribution to the phenotype of the trait and the allelic effect shows the effect of the female parent, Bay-0, alleles at the uncovered loci. If the value is negative the Bay-0 allele has a negative effect on Fv/Fm. The corresponding data is a comparison of the position of loci involved in leaf yellowing (Diaz et al. 2006).

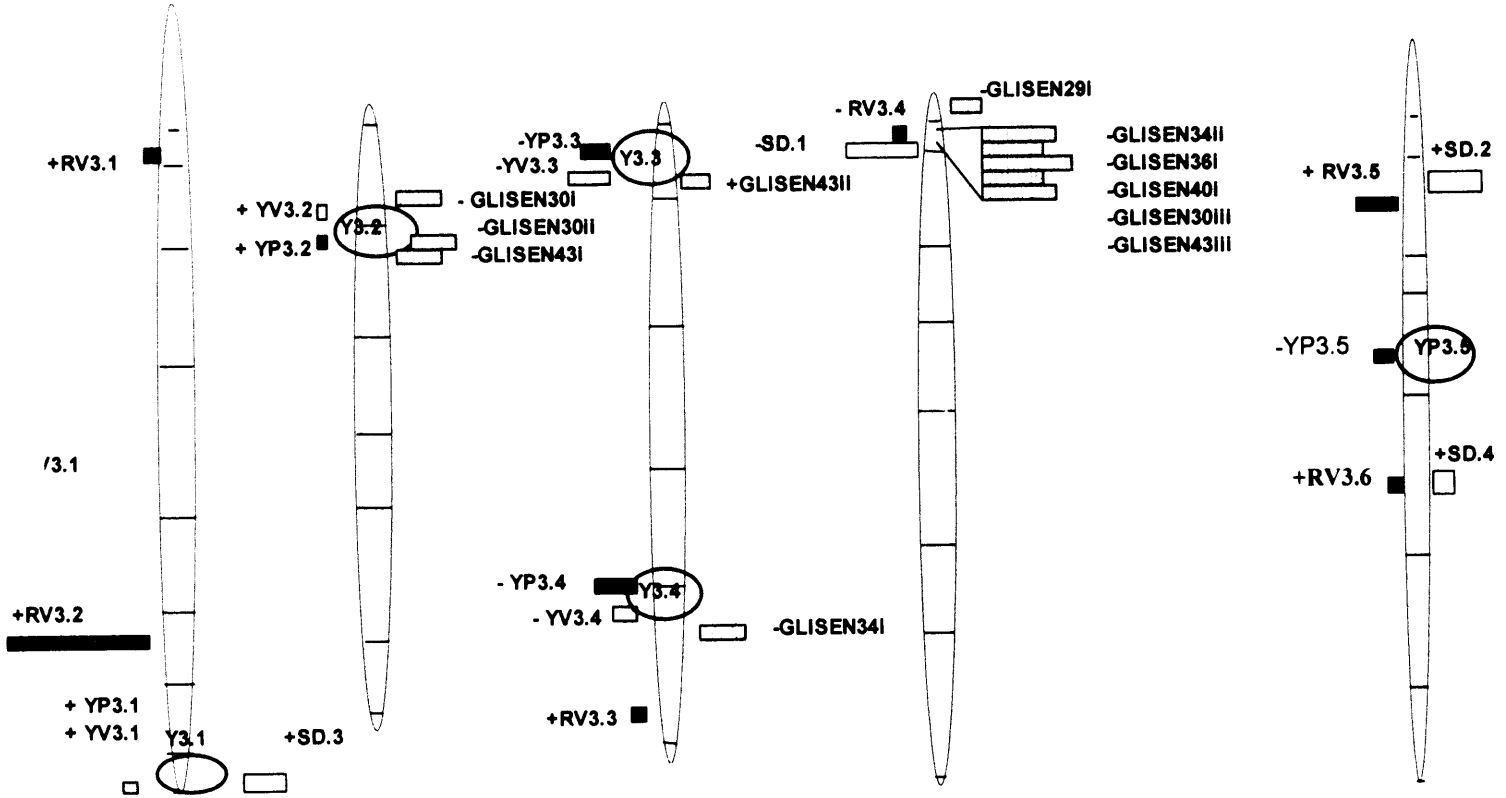


Figure 5.4. QTL for glucose induced senescence in low nitrogen conditions.

Data was taken from 180 recombinant inbred lines with a minimum replication of 15 plants for each RIL. The uncovered QTLs are positioned to the right of the chromosome map with the time points (DAP) in which they were detected. The size of the orange bars correspond to the R^2 value. QTLs marked to the left of the chromosomes locate the senescence associated loci visual yellowing (YV), visual reddening (RV), percentage yellowing (YP), percentage reddening (RP) or short day flowering (SD) (Loudet *et al*, 2002; Diaz *et al*, 2005).

The loci detected on Chromosome IV accounted for 8 -18.2% of the phenotypic variation and both Chromosome II and III accounted for 9-12% each.

5.2.3. QTL confirmation using near-isogenic lines (NILs)

Four pairs of NILs were used to confirm the presence of the two detected QTL on chromosomes II and IV. The QTL on chromosome II was found to be located between markers MSAT2.38 and MSAT2.5 and the locus on Chromosome IV was between markers MSAT4.39 and MSAT4.8. The near isogenic lines used to test the loci on Chromosome II were NILs 200-2 (Bay-0 allele), 200-1 (Shahdara allele) and 404-1 (Bay-0) and 404-4 (Shahdara). The NILs used for the loci on chromosome IV were 312-Bay-0, 312-Shahdara and 145- Bay-0 and 145- Shahdara. Plants were grown in low nitrogen or low nitrogen + 2% glucose conditions and were phenotypically assessed for maximum photosynthetic efficiency (F_v/F_m) and bolting, flowering and senescence development.

5.2.4. Analyses of near isogenic line pairs 145 and 312.

Two pairs of NILs (145 and 312) were used to confirm the QTL on chromosome IV. Figure 5.5 shows that in low nitrogen (LN) conditions no differences either between, or within the pairs of lines were observed. In LNG, however, the difference between 312 Bay-0 and 312 Shahdara is apparent. The decline in F_v/F_m started in all lines at DAP 32. In both 145 NILs and 312 Shahdara the decline was only slight between DAP 32-42 but in NIL 312 Bay-0 the decline was rapid with an average F_v/F_m of <0.4 at DAP 42 compared to >0.6 for NIL 312 Shahdara.

Figure 5.6 shows the visible appearance of the two pairs of NILs when grown in LNG. The difference is most obvious between 312 Bay-0 and 312 Shahdara as the Bay-0 allele is clearly conveying a more senescent phenotype than Shahdara which is showing senescence of the lower rosette leaves but new green leaves at the top of the rosette, similar to RIL 310 (Chapter 4). The opposite result appears to be seen in NIL 145 as 145 Shahdara appears more senescent due to the accumulation of anthocyanins but the F_v/F_m remained similar to the more green coloured 145 Bay-0 throughout. The difference in flowering phenotype can also be seen from Figure 5.6 with no visible flowering parts present on the selected plates in either 145 or 312 Shahdara.

Developmentally, more differences were observed in the timing and number of plants bolting and flowering within the two lines in both LN and LNG (Figure 5.7). In low nitrogen conditions flowering was notably different in both pairs of lines with the Bay-0 allele in both 312 and 145 exhibiting the accelerated phenotype over Shahdara. In LNG growth conditions NILs 145 Bay-0 and Shahdara remained similar in their flowering phenotype whilst 312 Bay-0 and Shahdara were different throughout the trial.

NIL 312 Bay-0 showed accelerated senescence with 100% of plants showing visible signs by DAP 30, whereas NIL 312 Shahdara took a further 12 days to reach 100%. In LN there was no significant differences in the senescence phenotypes within the two NIL pairs. The apparent discrepancy between the developmental graph, showing senescence occurring more quickly in LNG 312 Shahdara, whereas the photograph appears less senescent can be explained by the senescence phenotype of this NIL. NIL 312 Shahdara exhibits early senescence of the lower rosette leaves but then produces new leaves at the top of the rosette. NIL 312 Bay-0, however, initiates the

first visible symptoms of senescence slightly later but does not produce any new leaves.

These results demonstrate that glucose regulates genes within the locus uncovered by the QTL analyses as there are clear differences between plants treated with or without glucose. The difference in the senescence phenotype between lines 312 Bay-0 and 312 Shahdara is most pronounced confirming that the Bay-0 allele positively regulates glucose regulated senescence.

5.2.5. Analyses of near isogenic line pairs 200 and 404.

In LN growth conditions maximum photosynthetic efficiency (F_v/F_m) data revealed that 200-1 (Shahdara) showed the accelerated senescence phenotype compared to 200-2 (Bay-0) (Figure 5.8). This result was reversed in LNG after DAP 30. The differences between these two lines was slight, however, with an average F_v/F_m difference of 0.2 at the final time point.

In LNG, NIL 404-1 Bay-0 declined sharply between DAP 40-45 but was not significantly different at the end of the trial (data not shown). In LN treatment maximum photosynthetic efficiency remained similar between the two NILs and consistently high throughout the trial. In both pairs of NIL in LNG conditions but not LN, the Bay-0 allele produced the more accelerated senescence phenotype.

Figure 5.9 shows the pattern of development for the four NILs. The main difference was observed in the number of senescing plants in LN conditions with a clear difference between NILs 200-2 and 200-1. The discrepancy between the F_v/F_m data

and the senescence developmental data is the result of the senescence phenotype of this NIL which was similar to 314 and 145 Shahdara (see section 5.2.4).

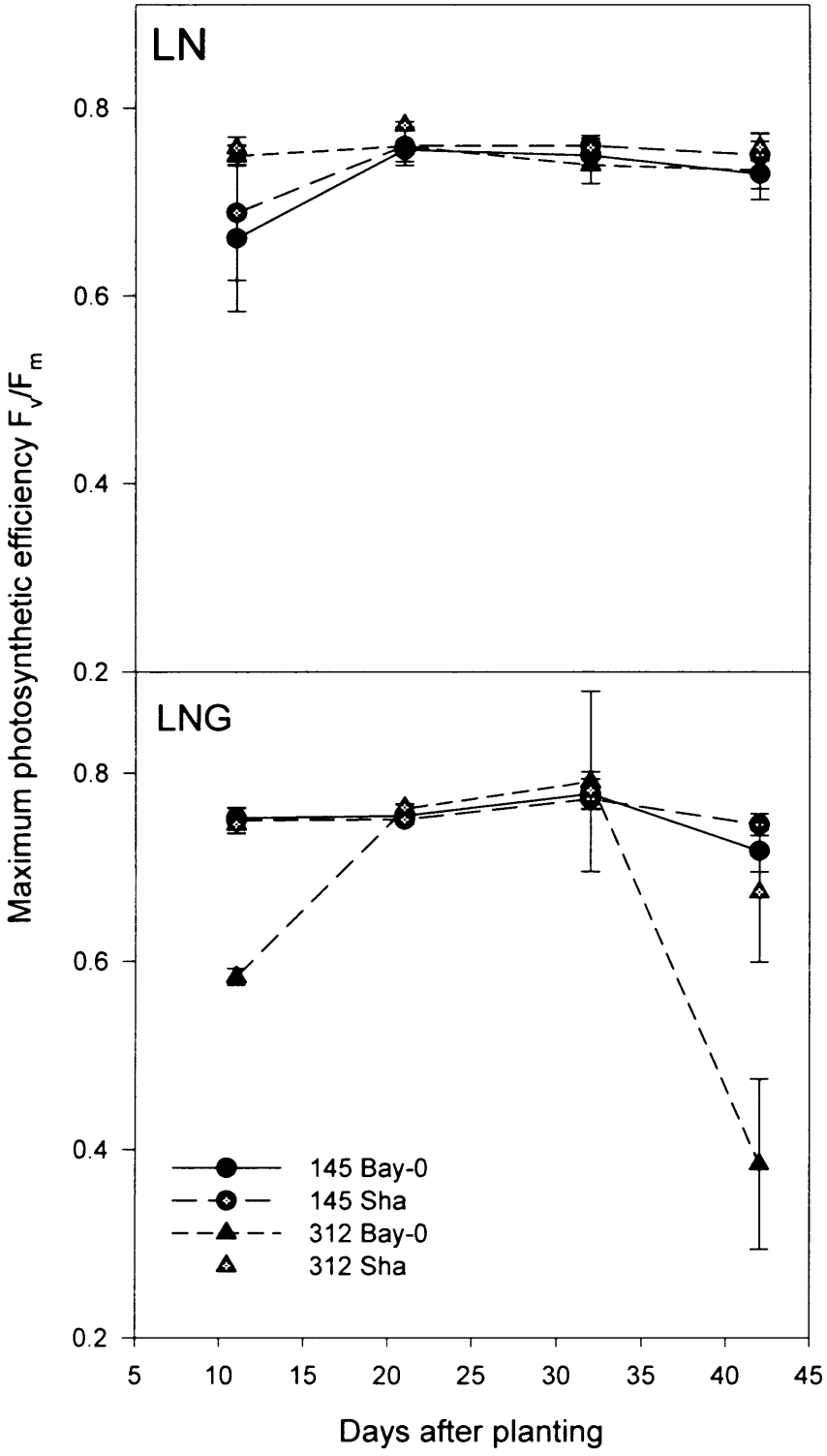


Figure 5.5. Maximum photosynthetic efficiency (F_v/F_m) of near isogenic lines (NILs) in response to low nitrogen (LN) and low nitrogen plus 2% glucose (LNG).

Data are based on a minimum of 40 plants per NIL and error bars denote standard error.

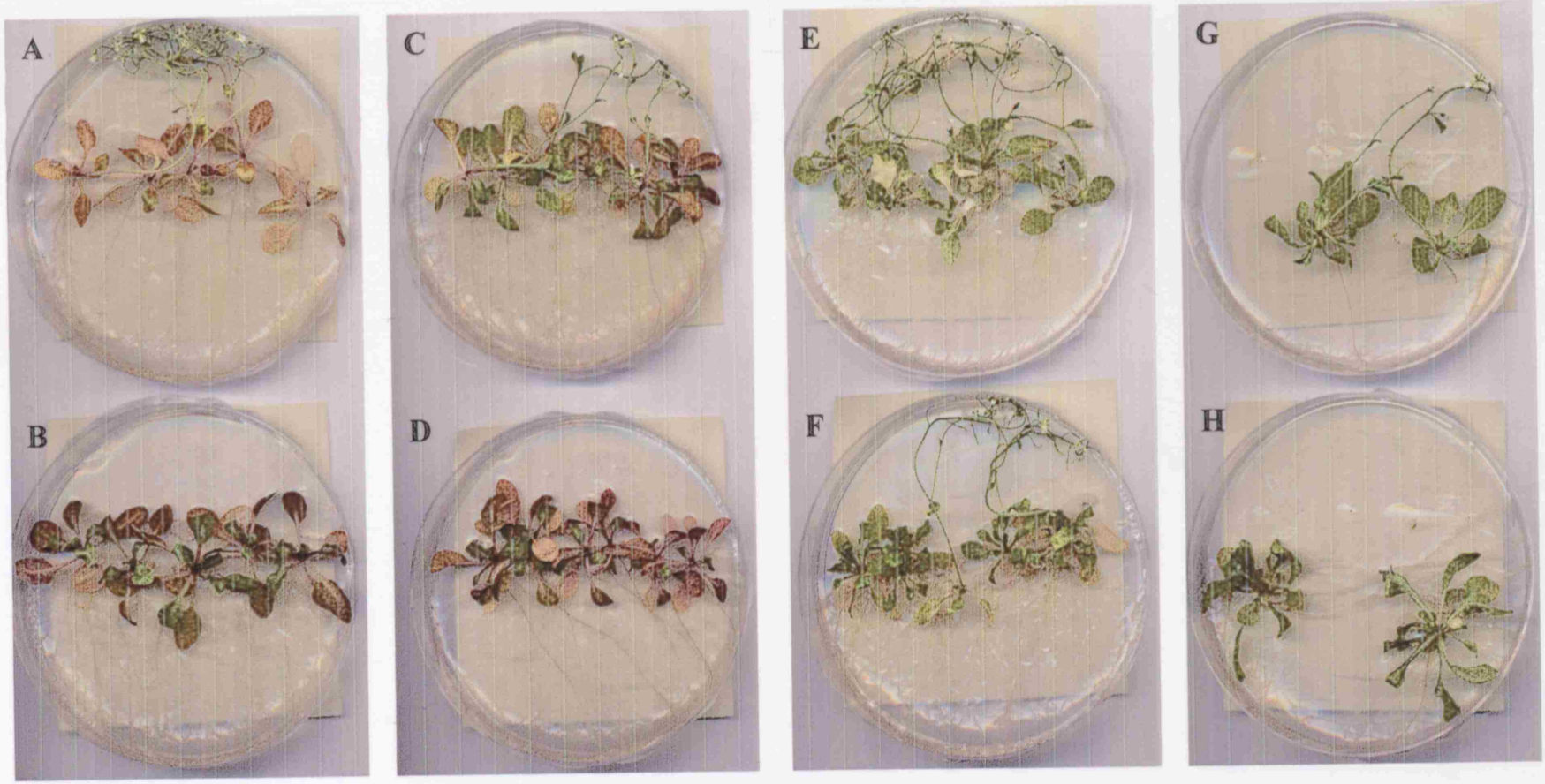


Figure 5.6. Near isogenic lines (NILs) response to treatment with low nitrogen (LN) or low nitrogen plus 2% glucose (LNG)

A: LNG NIL 312 Bay-0. B: LNG NIL 312 Shahdara. C: LNG NIL 145 Bay-0. D:LNG 145 Shahdara. E: LN NIL 312 Bay-0. F: LN NIL 312 Shahdara. G: LN NIL 145 Bay-0. H: LN NIL 145 Shahdara. All pictures taken DAP 35.

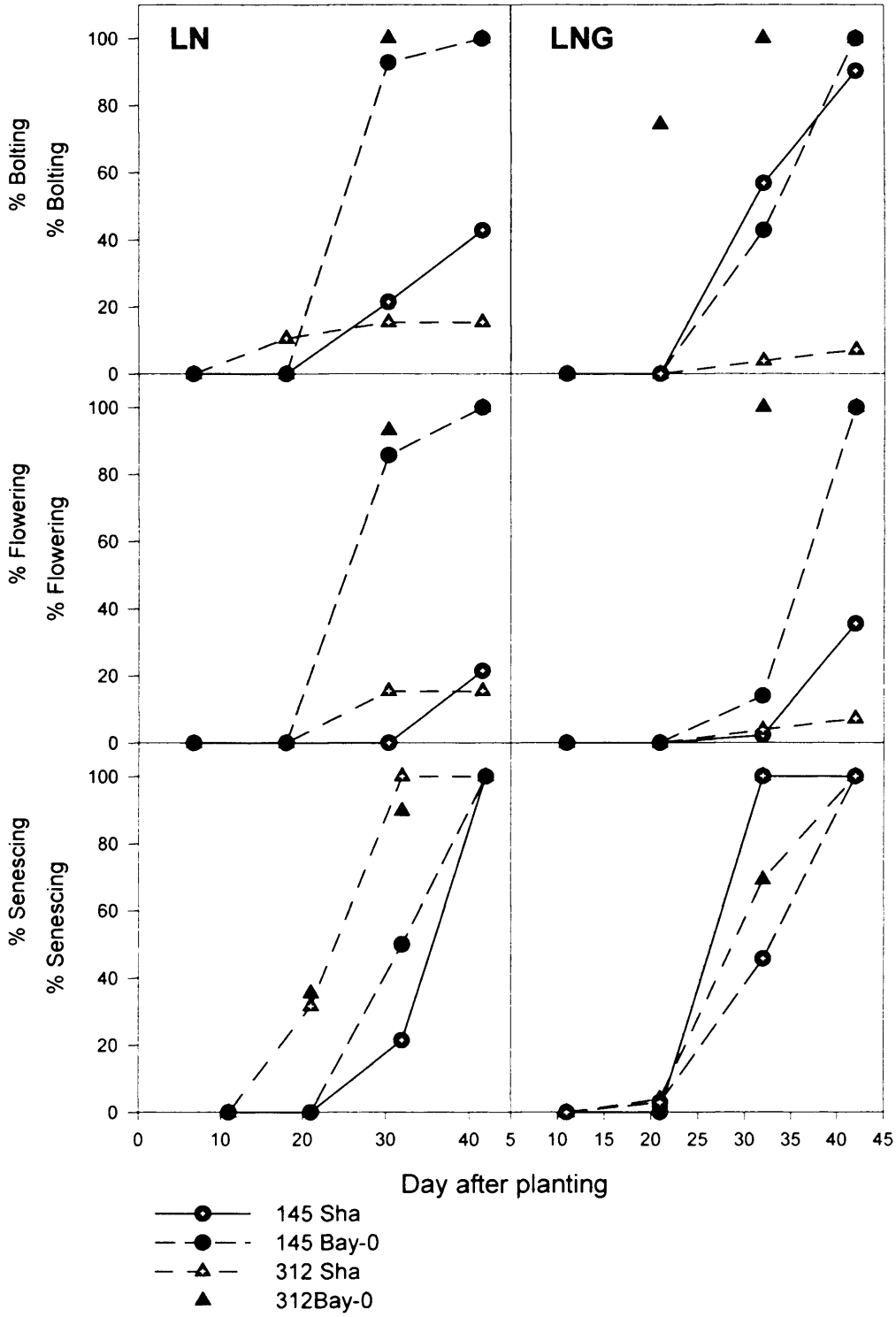


Figure 5.7. Development of near isogenic lines in response to low nitrogen (LN) or low nitrogen plus 2% glucose.

Data are based on the percentage of the total number of plants bolting, denoted by visual appearance of immature inflorescence, flowering by petal emergence, and senescing denoted by visual yellowing. A minimum of 40 plants per NIL were observed.

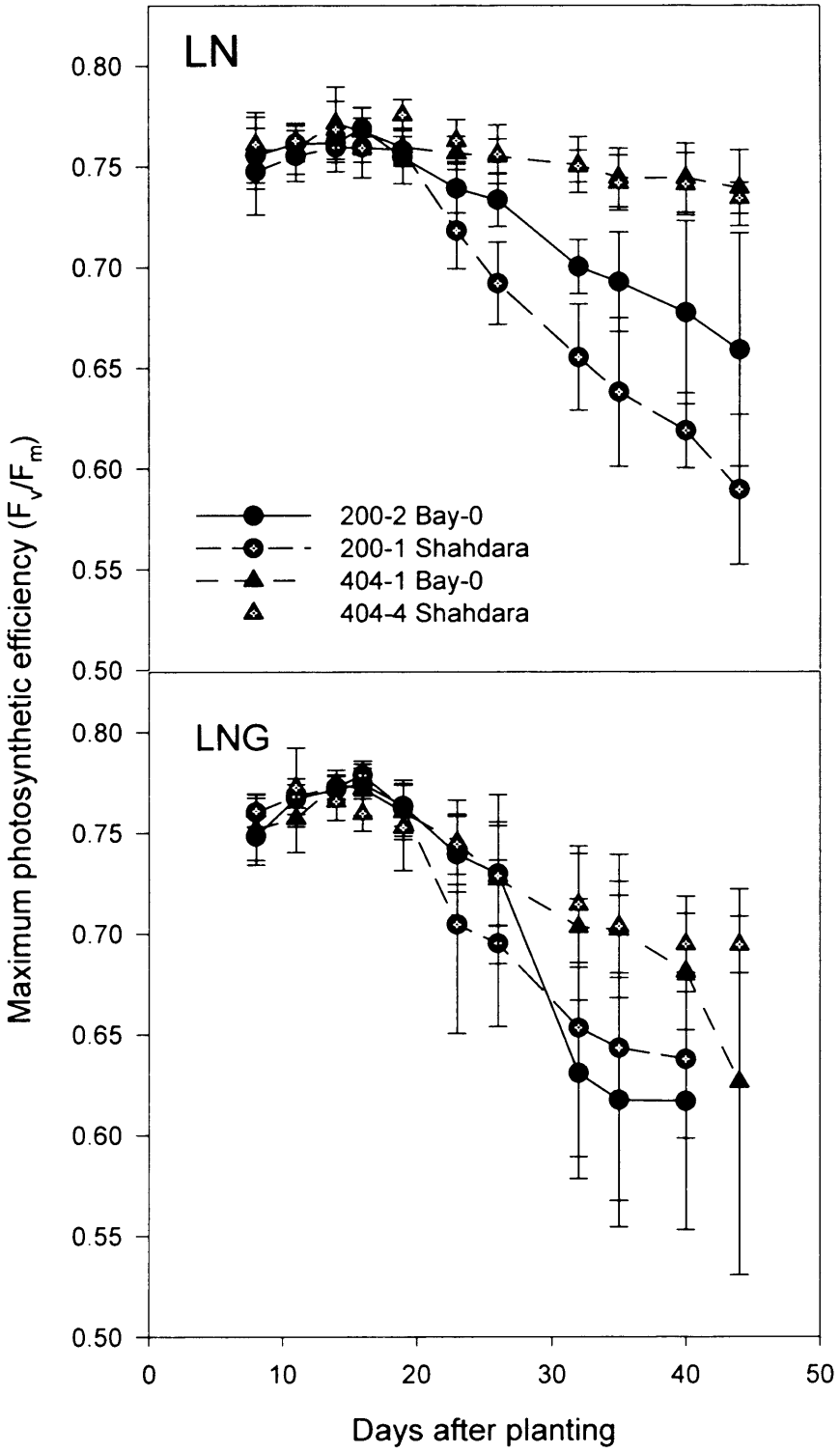


Figure 5.8. Maximum photosynthetic efficiency (F_v/F_m) of near isogenic lines (NILs) in response to treatment with low nitrogen (LN) and low nitrogen plus 2% glucose (LNG).

A minimum of 25 plants per NIL were tested and error bars represent standard deviation

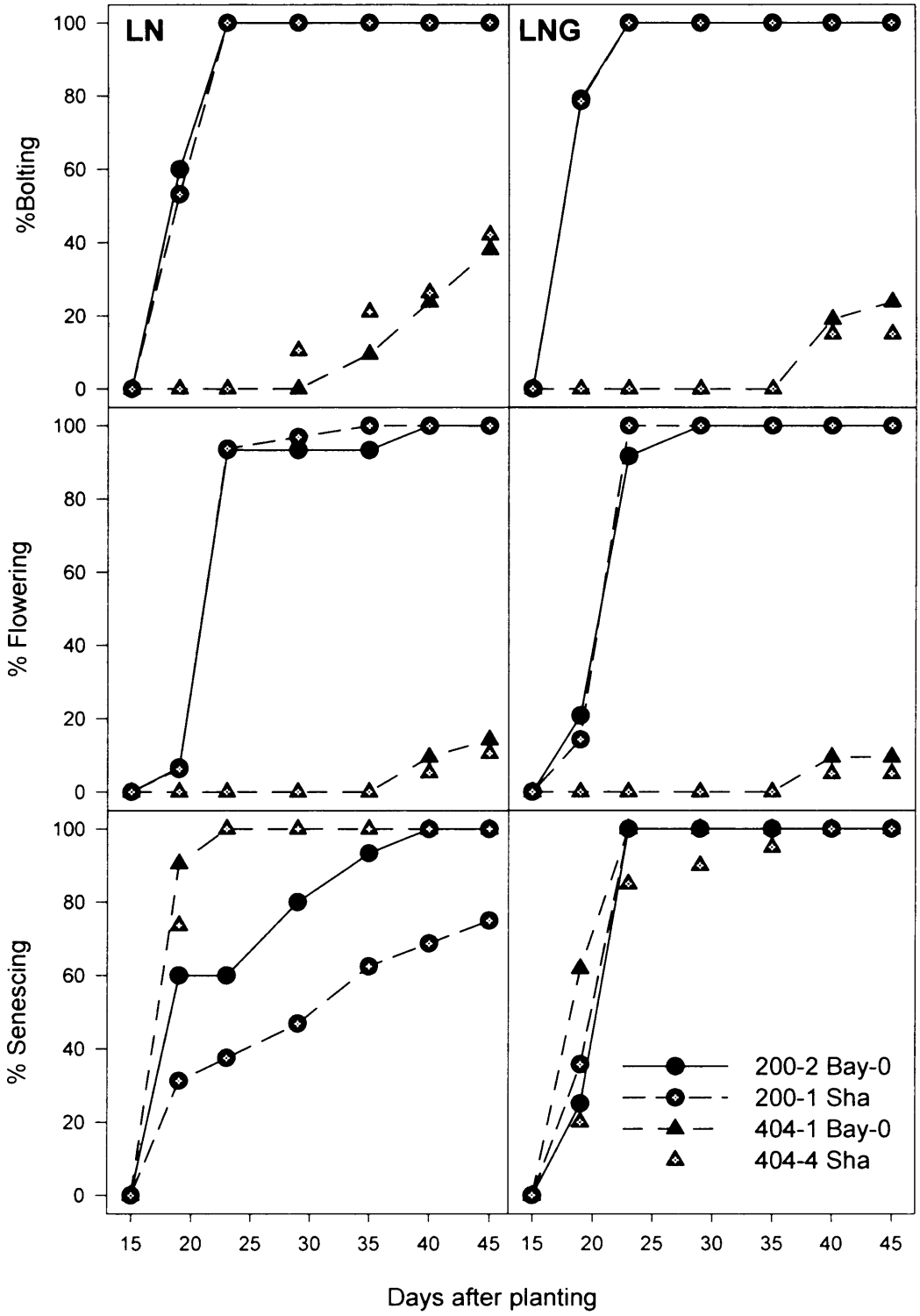


Figure 5.9. Development of near isogenic lines in response to low nitrogen (LN) or low nitrogen plus 2% glucose.

Data are based on the percentage of the total number of plants bolting, denoted by visual appearance of immature inflorescence, flowering by petal emergence, and senescing denoted by visual yellowing. The letter after the NIL number represents B: Bay-0 allele or S: Shahdara allele. A minimum of 15 plants per NIL were observed.

5.2.6. Response of freezing tolerance mutant, *sfr6*, to treatment with low nitrogen and low nitrogen plus 2% glucose

The *SFR6* gene maps to within the locus identified on Chromosome IV. As the expression of cold tolerance gene *COR15b* was strongly regulated by glucose (Chapter 4) it was hypothesised that *SFR6* could be the gene of interest by regulating senescence via a "feed-forward" loop (Masclaux et al. 2006; Appendix 4.3). Cold acclimatised plants accumulate sugars but do not exhibit the typical accelerated senescence phenotype. A mutation in the cold acclimation pathway such as in *sfr6* could, therefore, convey a more sugar sensitive phenotype in response to treatment with LNG. If *SFR6* was the gene of interest it was expected that the mutant plant would display an accelerated senescence phenotype compared to wild-type Col-0 on LNG medium.

Figure 5.10 shows that *sfr6* mutant plants grown in LNG media had a very low maximum photosynthetic efficiency (F_v/F_m) at the first time point, DAP 13, compared to both LN and LNG grown Col-0 and LN grown *sfr6*. Both Col-0 and *sfr6* declined more rapidly in their F_v/F_m phenotypes than their LN grown counterparts. Col-0 grown on LNG medium started to decline rapidly after DAP 33 whereas LNG grown *sfr6* started to decline seven days later at DAP 40. Col-0 plants had no photosynthetically active tissues at DAP 54 whereas *sfr6* retained an F_v/F_m value of >0.4 until the end of the experiment five days later. In a previous trial, however, the opposite result was observed and the mutant plants in LNG media had a lower F_v/F_m at the last time point. It must be noted, that the plants exhibited a wide range of variation in appearance and also F_v/F_m, particularly at the very beginning of the trial. The same degree of variation has since been observed in compost grown plants

indicating that the limitation is physiological and not a response to the treatment conditions. No differences between LN Col-0 and LN *sfr6* were observed; both retained a high F_v/F_m throughout the trial.

Visually the notable difference between *sfr6* and Col-0 was that *sfr6* accumulated anthocyanins and had fewer, elongated leaves than Col-0 on LNG medium (Figure 5.11). The anthocyanin accumulation was observed after DAP 25 and there was variation in its extent between individuals. The photographs presented were taken from the first trial of this mutant, in which the mutant displayed a generally more senescent phenotype than wild-type plants both visibly and from the F_v/F_m data.

No differences in the bolting and flowering phenotypes of the two lines (Col-0 and *sfr6*) in either treatment (LN and LNG) were observed (Figure 5.12). Indeed, LN and LNG *sfr6* were identical in the number of plants bolting and flowering throughout the trial.

5.2.7. Contribution of Bay-0 and Shahdara alleles in the ten latest senescing RILs

The chromosomes of the ten latest senescing RILs were represented diagrammatically to show the contribution of Bay-0 and Shahdara alleles along them. On chromosome IV (Figure 5.13) all of the RILs were found to have Shahdara alleles within the locus identified at the top of this chromosome, between markers MSAT4.39 and MSAT4.8. No other similarities were observed between them on the other chromosomes (data not shown)

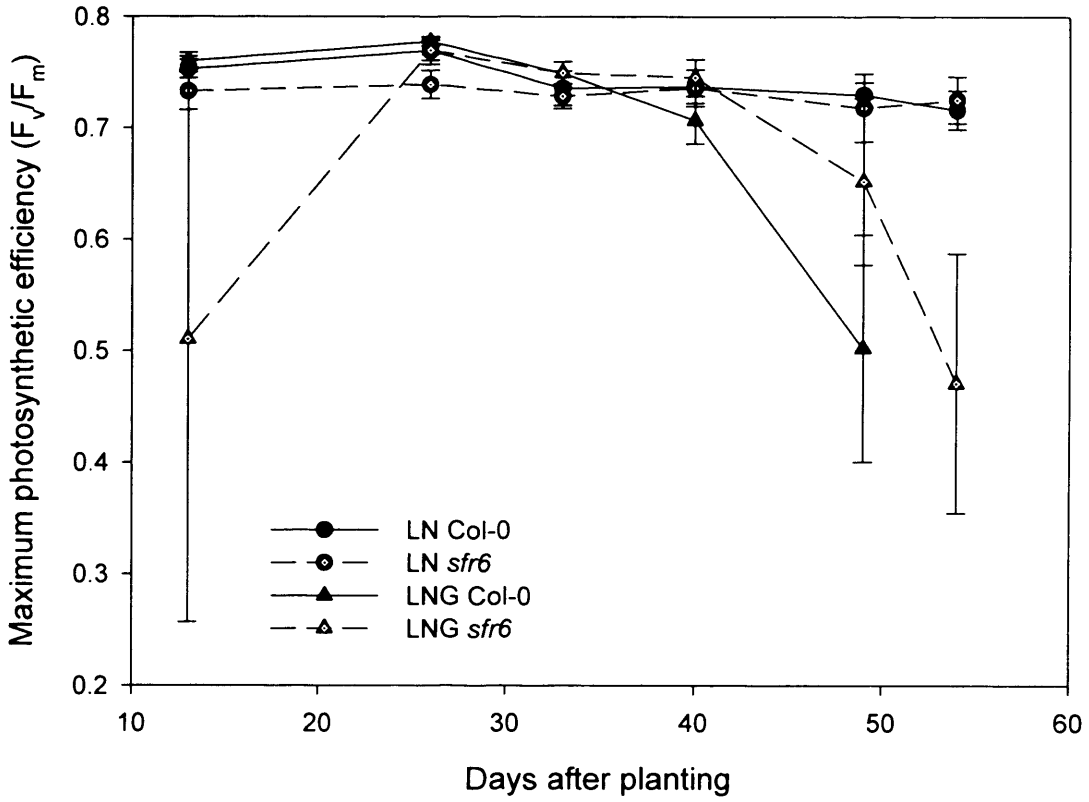


Figure 5.10. Maximum photosynthetic efficiency (F_v/F_m) of *sfr6* mutant plants and wild type Col-0.

Data is based on a minimum of 40 plants per accession. Error bars denote standard deviation

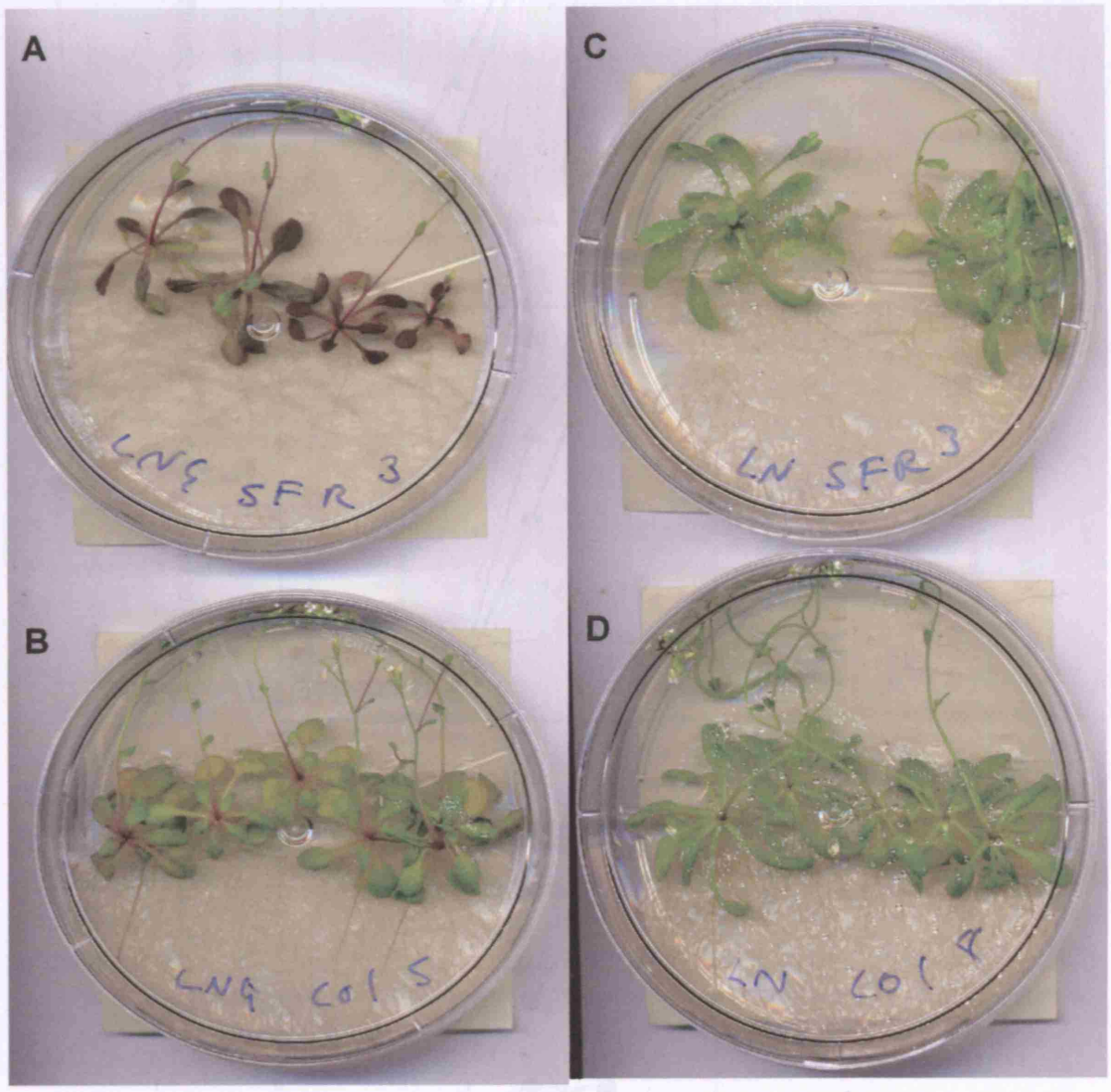


Figure 5.11. Cold tolerance *sfr6* mutant plants and wild type Col-0 plants in response to treatment with low nitrogen (LN) or low nitrogen plus 2% glucose (LNG).

A: *sfr6* mutants plants in response to LNG, B: Col-0 plants in response to LNG, C: *sfr6* mutant plants in response to LN and D: Col-0 plants in response to LN.

Pictures were taken 35 days after planting.

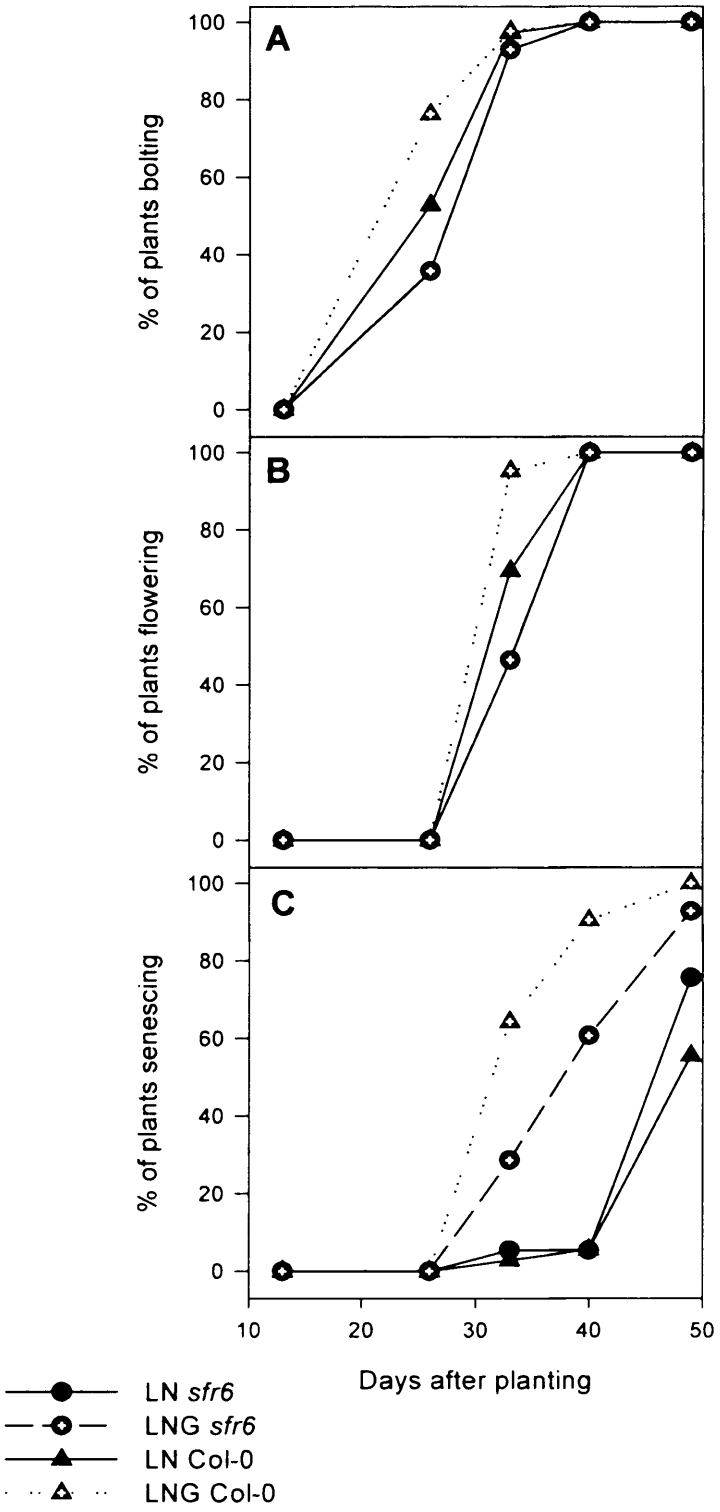


Figure 5.12. Development of *sfr6* mutant plants and wild type, Col-0, in response to treatment with low nitrogen (LN) or low nitrogen plus 2% glucose (LNG).

Data represent the percentage of the total number of plants A: bolting, B: flowering and C: senescing per line. Bolting was denoted by the first appearance of the immature inflorescence, flowering was denoted by the appearance of flower petals and senescence by visual yellowing.

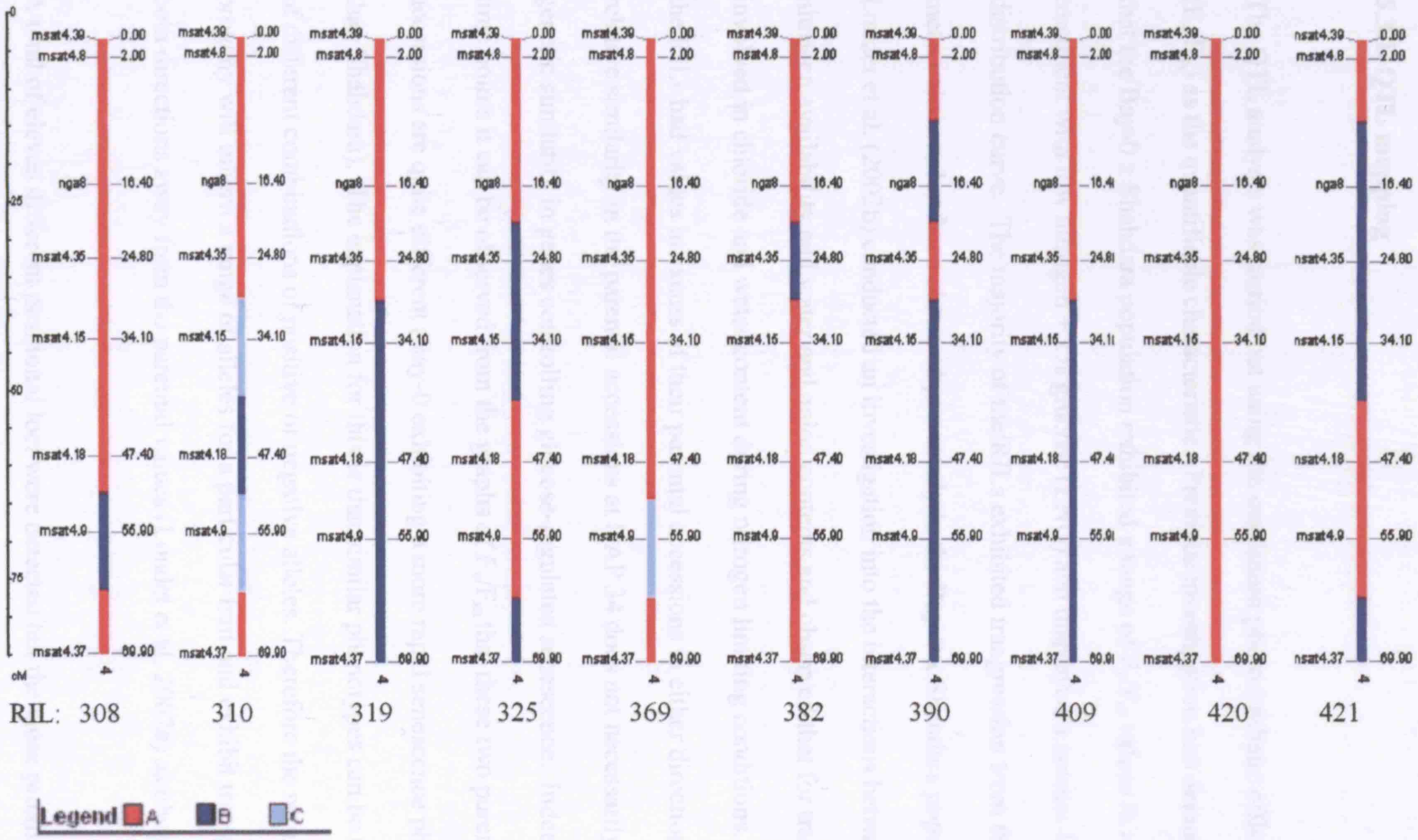


Figure 5.13. Diagram of chromosome IV of the ten least senescent recombinant inbred lines (RILs) showing the composition of Bay-0 and Shahdara alleles

Red bands (A): Shahdara alleles, dark blue bands (B): Bay-0 alleles, light blue bands (C): heterozygous region.

5.3 Discussion

5.3.1. QTL mapping

The QTL analysis was carried out using the maximum photosynthetic efficiency (F_v/F_m) as the quantifiable characteristic. Previous investigation had demonstrated that the Bay-0 x Shahdara population exhibited a range of F_v/F_m values in response to treatment with low nitrogen + 2% glucose (LNG) and displayed a normal frequency distribution curve. The majority of the RILs exhibited trangression from the parental mean values as has been observed previously in the Bay-0 x Shahdara population. Loudet et al. (2002b) conducted an investigation into the interactions between nitrogen availability and water and anion contents and observed that for traits involved in chloride and water content during nitrogen limiting conditions, most of the RILs had values in excess of their parental accessions in either direction. The relative similarity in the parental accessions at DAP 34 does not necessarily reflect genetic similarity in genes controlling glucose-regulated senescence. Indeed, at later time points it can be observed from the graphs of F_v/F_m that these two parental accessions are quite different (Bay-0 exhibiting a more rapid senescence phenotype than Shahdara). The explanation for this is that similar phenotypes can be the result of different combinations of positive or negative alleles. Therefore the resulting progeny will inherit a range of alleles for a particular trait and exhibit transgression in both directions away from the parental values (Loudet et al. 2002a) as observed here.

A total of eleven different positional loci were detected but the close proximity and consistency of those detected on Chromosomes II and IV, respectively, means that they almost certainly relate to the same genes of interest. Two loci were detected on

Chromosome III but they were spatially separate. The total percentage of phenotypic variation explained by the QTL was between 8% (GLISEN 29) and 18% (GLISEN 40). This is consistent with the values observed by Diaz et al. (2005) of yellowing and anthocyanin accumulation which ranged between approximately 2-12% for yellowing and 2-33% for anthocyanin accumulation.

The R^2 value denoting the contribution of the highest scoring QTL to the total phenotypic variance was 18.21% which is considerably higher than values reported for many other studies. A study by Loudet et al. (2003) of the interaction between water and anion content with nitrogen availability identified 45 QTLs of which 44, individually, had an R^2 value of <16%. The relatively low contribution of most of the individual loci can be attributed to the polygenic basis and sensitivity to environmental stimuli of the trait (Diaz et al. 2005) or may be attributed to digenic interactions (Farouk et al. 2005). That a trait can be governed by many genes, but only a few loci, is not unusual. For example, in a study in to the starch and protein composition of maize kernels Dudley and Lambert (1992) calculated that up to 173 genes were responsible for the phenotype but subsequent QTL analyses identified that only 6 or 7 QTLs were responsible for explaining some 65-66% of the variation.

The allelic effect of each QTL, was negative in all, except GLISEN 43ii. This means that the Bay-0 allele had a negative effect on maximum photosynthetic efficiency (F_v/F_m) in all the QTLs except GLISEN 43ii. This is supported by all the data from the characterisation studies (Chapter 4), which shows that Bay-0 has a lower F_v/F_m than the male parent Shahdara. Furthermore, a comparison of the marker genotype of the nine RILs that maintained the highest F_v/F_m at the latest time point (DAP 40) revealed that all ten had the Shahdara alleles within the identified locus at the top of

chromosome IV, between markers MSAT4.8 – MSAT 4.39 (Figure 5.13). This strongly indicates that the Shahdara alleles at these loci are responsible for conferring prolonged vegetative growth. The gene(s) within the QTL at the top of chromosome IV cannot be solely responsible for controlling sugar-regulated senescence, however, as if this were the case Shahdara would exhibit a delayed senescence of equal magnitude to the ten latest senescing RILs, which it does not. The locus must therefore interact synergistically with at least one other locus to produce the delayed senescence phenotype. Regulation of complex traits by multiple loci has been observed in a number of studies. In the control of flowering time in the L*er*/Cvi population two loci, FLF and FLG from Cvi, are known to act synergistically to incur a late flowering phenotype (Alonso-Blanco et al. 1998). QTL analysis of submergence tolerance in RILs of rice (*Oryza sativa*) uncovered one major QTL, so called QTL_{ch9}, and two secondary QTLs, QTL_{ch5} and QTL_{ch7}, which also affected the trait (total shoot elongation before submergence). It was observed that when QTL_{ch9} was expressed QTL_{ch5} and QTL_{ch7} were not. A possible explanation for this is that both QTL_{ch5} and QTL_{ch7} are part of the submergence tolerance pathway which are rendered redundant in the presence of QTL_{ch9}. However, it was observed that the submergence tolerance of RILs carrying all three QTL (F_{QTLch9}, F_{QTLch5}, F_{QTLch7}), as opposed just QTL_{ch9} (F_{QTLch9}), was superior, implying digenic interactions between the three QTL (Toojinder et al. 2003).

The NIL data confirmed the QTL on chromosome IV by clearly showing the negative effect of the Bay-0 allele on F_v/F_m when grown in LNG. Both visually and the F_v/F_m data showed that in LN treatment little difference between the Bay-0 / Shahdara alleles was observed for either NIL except that the Shahdara allele produced a slightly more yellowed phenotype in LN conditions. This is supported by Diaz et al. (2006)

who observed that yellowing in NILs carrying a Shahdara allele was 5-fold higher than NILs carrying a Bay-0 allele at loci conferring leaf yellowing (YP3.4 and YV3.4). NILs 312 Bay-0 and Shahdara were markedly different in their senescence phenotypes in LNG and therefore provide strong evidence to support the QTL position and effect on Chromosome IV. The accelerated senescence phenotype of the 312 Bay-0 can be seen both from the F_v/F_m data and the photographs. Senescence was actually first observed in the lower rosette leaves of 312 Shahdara but this NIL continued production of new leaves at the top of the rosette similar to that of RIL 310 (Chapter 4). Flowering was also repressed in 312 Shahdara and also 145 Shahdara in both treatments. This strongly suggests that flowering in Shahdara is dependent on nitrogen status, as previous studies have cited this RIL as being early flowering (Werner et al. 2005; Luquez et al. 2006). As glucose had little (or no) effect of relieving this repression it implies that the signal to initiate flowering is independent of sugar and mediated by nitrogen alone. It is therefore possible that the continued production of new leaves in late senescing RILs and parental accessions, such as Shahdara and 310, is due to the retardation of flowering in response to unfavourable nutritional conditions.

NIL 145 Shahdara had fewer green leaves than NIL145 Bay-0 and a greater accumulation of anthocyanins when grown in LNG, but it maintained a higher F_v/F_m throughout. This is because anthocyanin accumulation usually precedes yellowing which is a symptom of chlorophyll catabolism (Diaz et al. 2006). A protective role of this pigment has been suggested to act by absorbing surplus light that could result in the build up of ROS due to the degrading chloroplast and subsequent disruption of the electron transport chain during senescence.

5.3.2. Candidate genes

The position of the identified locus is between 0.01 – 16.4 cM at the top end of chromosome IV. The average position of the 6 loci is 6.35 cM. The locus lies between genes At4g00210 (89498 bp) – At4g00940 (407010 bp) (http://www.arabidopsis.org/servlets/mapper).

The position of the *SFR6* gene was unknown when the trial was initiated, but it had been localised to being 16.6 cM on the top of chromosome IV (A. Wingler; personal communication) which makes it a reasonable candidate gene. A second nomination is gene At4g03290 which is also involved in cold tolerance but is more accurately located 8 cM from the top of chromosome IV.

Sugars are required for cold tolerance to increase the solute concentration of the cell and protect against severe dehydration, the principally destructive property of freezing (Fowler and Thomashow, 2002). Despite the increase in sugar contents, however, cold-acclimatised plants are less sugar sensitive during senescence (Masclaux-Daubresse et al. 2007). Therefore as *sfr6* is impaired in cold acclimation, it could, theoretically, be more sugar sensitive. As had been observed for all accessions previously tested (except RIL 310), such Ws-2 (Pourtau et al. 2006) and L*er*-0 (Pourtau et al. 2004) plants grown on LNG medium senesced more quickly than their LN grown counterparts. The response of *sfr6* to treatment with LN or LNG compared to the wild type, Col-0, was, however, largely inconclusive as a variable response of *sfr6* to glucose was found in different experiments. The plants used in this trial were all grown at 22°C and so it is possible that *sfr6* may exhibit reduced sugar sensitivity

at low temperatures. This line of enquiry is to be investigated as a follow on to this study (A. Wingler, personal communication).

The method by which a gene such as *SFR6* could regulate senescence would involve a "feed-forward" loop; as genes involved in cold tolerance can be induced by glucose (Masclaux-Daubresse et al. 2007). Furthermore, the observation that *COR15b* was strongly regulated by glucose in 310 (Chapter 4) was suggestive that the specific gene of interest on chromosome IV could be involved in cold tolerance.

Senescence associated gene 21 (*SAG21*) (AT4G02380), is located within the locus on chromosome IV. This gene is maximally expressed before senescence is visible and expression then decreases (Weaver et al. 1998). This is indicative that *SAG21* may play a role in regulating other SAGs as senescence progresses (Wang et al. 2000). The expression of *SAG21* in response to dark incubation and hormonal treatments is unique compared to other known SAGs. Detached leaves that are placed in the dark tend to senesce very quickly and this method has been used to investigate senescence mechanisms in a number of studies (Buchanan-Wollaston et al. 2005; Becker and Apel, 1993). However, unusually, *SAG21* is not expressed in detached leaves when placed in the dark but detached leaves placed in light conditions do express this gene (Weaver et al. 1998). This implies that darkness inhibits expression. That light is required for expression indicates that sugars could act as a signal for the expression of this gene making it a strong candidate gene and indeed it has been observed that expression is upregulated in response to glucose treatment (Pourtau et al. 2006). It would be interesting to test whether the application of glucose can overcome dark induced repression of *SAG21*. Intriguingly, considering its lack of response in dark incubated attached leaves, *SAG21* is strongly expressed by whole plant incubation in

darkness. If *SAG21* is regulated by sugars it is possible that the expression observed when the whole plant was dark incubated was the result of starch breakdown and resource allocation, allowing stored carbohydrates to be released and mobilized to allow the necessary senescence responses to occur.

SAG21 is responsive to cold and ROS. The role of glucose in regulating genes involved in cold tolerance has been discussed previously (see also Appendix 5.2) but its response to reactive oxygen species is also supportive of a role in glucose-regulated senescence. High levels of glucose within leaf cells can both enhance production of ROS and reduce production through fuelling ROS scavenging molecules (Couee et al. 2006). Sugars may protect against photo-oxidative damage by feeding NADPH producing pathways, such as the oxidative pentose-phosphate (OPP) pathway which contribute to the scavenging of ROS. High sugar levels can contribute to ROS production by, counter-intuitively, repressing photosynthesis. This occurs because $NADP^+$ is poorly re-cycled during the down regulation transition leading to excessive electron transfer (Couee et al. 2006). This presents an interesting relationship between sugars and cold tolerance. As has been discussed previously, sugars accumulate during chilling to protect the plant from dehydration. However, sugar accumulation may trigger ROS production and certainly an accumulation of ROS is observed during chilling (Couee et al. 2006). It is possible, then, that *SAG21* which responds to both cold and ROS could, via sugars, regulate the relationship between sugars and ROS; ensuring maximum protection with minimum damage.

A large number of genes, of known function, within the locus on chromosome IV are associated with ubiquitin (At4g05310) or polyubiquitin (UBQ10). Polyubiquitin is known to be active in senescent tissues (Belknap and Garbarino, 1996) which is

unsurprising as its function is in proteolysis which is essential in the recycling of nitrogen during senescence. Ubiquitin is covalently attached to many proteins within the chloroplast. Proteolysis of proteins via ubiquitin requires three enzymes: Firstly, ubiquitin-conjugating enzyme (E1) which bonds to the ubiquitin protein and catalyses its ATP-dependent activation. A second enzyme (E2) then acts as a carrier protein which can either transport the activated ubiquitin directly to the target protein, or it may (if necessary), transport it to the final enzyme ubiquitin protein ligase (E3) to form a bond with the target protein (Belknap and Garbarino, 1996).

Increases in transcription rate and patterns have been observed during senescence in *Nicotiana* and Arabidopsis (Genschik et al. 1994) and potato (Garbarino et al. 1995) and treatment with senescence promoting hormones, such as ethylene, was also observed to enhance transcription of ubiquitin genes (Belknap and Garbarino, 1996).

Although the nature of the relationship between ubiquitin and senescence has not yet been elucidated it is highly likely that these proteins have an active role in regulating senescence. Their principal function is probably in freeing stored nitrogen for remobilisation to the reproductive tissues but other, more diverse functions could exist. The high sugar and low nitrogen conditions experienced in the LNG-treated plants would probably have resulted in the down-regulation of genes involved in photosynthesis and consequently the proteolysis of some photosynthetic proteins. It is, therefore, possible that ubiquitin mediated this reaction and was directly stimulated by glucose content.

Flowering gene *FRIGIDA* (At4g00650) maps to 7 cM from the top of Chromosome IV. This gene is responsible for controlling vernalization-dependent flowering in

winter annual accessions of Arabidopsis via the action of a second gene, Flowering Locus C (*FLC*). *FLC* is a floral inhibitor regulated by *FRIGIDA* (Werner at al. 2005) and accessions that do not require vernilization to flower usually exhibit a null mutation of one or both of these genes (Michaels et al. 2004). Summer annuals are often the result of allelic variation of *FLC*. The Landsberg erecta (L*er*) accession bears an insertion of a transponson-related sequence within a large intron that is required for correct transcription (Werner et al. 2005). Consequently, the *FLC* alleles only have a limited response to *FRIGIDA* in this accession. Interestingly, this same reduced response to *FRIGIDA* has been observed in Shahdara which has often been cited as being an early flowering accession (Luquez et al. 2006; Werner at al. 2005). However, as the results presented in Chapter 4 show, the early flowering phenotype of Shahdara is dependent on nitrogen status as in either LN or LNG conditions flowering was slower than the majority of RILs tested indicating a regulatory role of nitrogen on *FLC* expression. The flowering phenotype of RIL 310 is vernalization dependent and whole rosette senescence occurs following early flowering if this treatment is applied (A. Wingler, personal communication). This implies that senescence is dependent on flowering and as Shahdara has a reduced function *FLC* allele, but the Bay-0 allele remains functional, it would be expected that the resulting RILs would display a range of phenotypes as was observed. Furthermore, Loudet et al. (2002) verified that Bay-0 actually has a recessive allele of *FRIGIDA* whereas Shahdara is fully functional in this gene, adding further support to the possibility that the observed variation is due to the inheritance of different alleles of *FRIGIDA* and *FLC*. The senescence phenotypes could therefore be more dependent on flowering, or specifically the expression of *FRIGIDA*, rather than glucose, to instigate senescence and that the role of glucose is actually to regulate floral initiation.

Nitrogen metabolism gene, *GLB1* (At4g01900.1) is located within the locus on chromosome IV. *GLB1* is a glutamine synthetase regulator and has been postulated to have a role in signalling the carbon:nitrogen status of Arabidopsis (Hsieh et al. 1998). Genes involved in nitrogen metabolism are known to be up-regulated by high levels of glucose (Pourtau et al. 2006) and during developmental senescence (Buchanan-Wollaston et al. 2005). An explanation for this is that the rate of general metabolism is increased in the presence of ample glucose (Price et al. 2004), but during senescence a number of complex reactions involving nitrogen metabolism occur. An example of this is the conversion of amino acids after proteolysis for transport via the phloem. The majority of nitrogen that is remobilized is in the form of glutamine and three genes involved in glutamine synthetase (*GLN1;4*, *GLN1;1*, *GLN1;3*) were up-regulated in response to LNG growth conditions (Pourtau et al. 2006) but not in response to dark induced senescence (Buchanan-Wollaston et al. 2005). The repression of *GLB1* by dark incubation could be overcome by the application of 3% sucrose, but the application of organic nitrogen was unable to reduce expression strongly suggesting a role in carbon:nitrogen regulation. As the expression of *GLB1* is regulated by light and metabolism (Hsieh et al. 1998) and as growth in LNG conditions produced a dis-equilibrium in carbon:nitrogen ratio, it is plausible that this gene was responsible for signalling this status during senescence. Furthermore, the position of *GLB1* is situated 10.8 cM from the top of Chromosome IV (Hsieh et al. 1998) which is within the uncovered locus further justifying its nomination as a candidate gene.

To try to prove that one of the proposed candidate genes is the specific gene of interest a number of approaches could be taken. Firstly gene sequencing would reveal polymorphisms of the proposed gene between Bay-0 and Shahdara (sequencing of the

SFR6 gene is currently underway). If polymorphisms could be identified, a transformation of the Shahdara allele into the Bay-0 background would allow the response of the resulting transgenic plants to LNG conditions to be tested. If senescence was delayed, in the transgenic plant compared to the parent Bay-0, it would strongly indicate that the nominated gene is the gene responsible for regulating glucose-induced senescence.

A second approach would be to use mutant plants, such as a *GLB1* or *UBQ10* mutants to test the response to treatment. The problem with this approach is that mutants would probably have to be identified in the Shahdara background, as the Shahdara allele confers the unique late senescence phenotype. Mutant collections are, however, not available for Shahdara. An alternative approach would be to use RNA interference in order to create plants with strongly reduced expression of the candidate gene in Shahdara. This would, however, be labour intensive.

Fine mapping could be used for more accurate localisation of the QTL, reducing the number of possible candidate genes but this would be time consuming and labour-intensive.

Chapter 6

General Discussion

6.1 The metabolic regulation of leaf senescence

Senescence was accelerated in the Arabidopsis accessions Bay-0 and Shahdara, and in most lines of the RIL population, when plants were grown with low nitrogen and glucose (LNG) in the growth medium. Accelerated senescence was not observed when glucose was added to high-nitrogen medium or on low-nitrogen medium in the absence of glucose. This observation is in agreement with all accessions previously tested (Pourtau et al. 2004; 2006) and is thus conclusive that this treatment uniquely regulates senescence. However, exceptions were found in some lines of the Bay-0 x Shahdara RIL population (see below). It should be noted that, in contrast to the wild-type accessions, the RILs have not evolved in nature and may therefore show untypical characteristics that may impair fitness under natural conditions.

By testing the response of Arabidopsis plants to the two osmolytes, mannitol and sorbitol, it was confirmed that accelerated senescence was not the result of osmotic stress. Furthermore, the observation that the induction of *SAG12*, a definitive marker of senescence, was induced by growth in the presence of glucose is indicative that glucose-induced senescence is representative of developmental senescence.

The observation that sugars, generally, accumulate during senescence rather than decreasing, strongly contradicts the theory that sugar starvation could be responsible for causing leaf senescence. The accumulation could be the result of reduced utilization of carbon for producing amino acids. Evidence for this was suggested by the phenotype of RIL 310 as this line had an unusually high content of amino acids (Diaz et al. 2005; Appendix 4.6) and, consequently, reduced leaf sugar contents and had a late senescing phenotype.

It is probable that hexokinase-1 acts as the initial signal as hexokinase-1 mutants exhibit delayed senescence when grown in LNG growth medium (Pourtau et al. 2006) and the non-metabolisable glucose analogue 3-O-methylglucose, at the same concentration as glucose treatment (111 mM), does not induce senescence (A. Wingler, personal communication). Senescence is not induced if whole plants are incubated in the dark, but shading of individual leaves induces senescence in the shaded area (Weaver and Amasino, 2001). This is probably an adaptation to ensure that energy and reserves are not wasted maintaining an organ that is not photosynthetically efficient. In whole plant darkness sugar depletion will rapidly occur so that there is an inadequate supply of energy to either continue promoting reproductive development or produce new leaves. Darkening of whole plants therefore appears to induce a form of stasis in that development (including senescence) stops until light is received and sugars can be produced again. This is highly indicative that individual leaf senescence is not caused by sugar starvation. Furthermore, when considered with the observed accelerative effect of glucose it seems probable that glucose may instigate senescence by acting as a molecular signal for the nutritional status of the plant.

6.2 Natural variation in the metabolic regulation of leaf senescence

Variation in the regulation of senescence by glucose was observed in lines of the Bay-0 x Shahdara population. While Shahdara showed a slightly weaker response to glucose than Bay-0, considerable transgression was found in the RILs. For example, RIL 45 exhibited a considerable acceleration in leaf senescence in the presence of glucose, whereas RIL 310 did not show the typical senescence response to glucose. In addition to the F_v/F_m values, the ratio of chlorophyll a:b was found to be representative of the senescence phenotypes of the different RILs. The later senescing lines such as 310 and Shahdara retained a high ratio, whereas the early senescing lines such as Bay-0 and 45 had a lower ratio. As the drop in chlorophyll content usually occurs as a result of a large decrease in chlorophyll b (Oh et al. 2000), it is probable that the later senescing lines were able to retain the ratio between the two chlorophylls.

RIL 310 plants were atypical in their response to LNG growth conditions. Whole rosette senescence was delayed as new leaves continued to be produced at the top of the rosette and flowering was only observed if exogenous sugars had been provided. The expression of selected genes confirmed these observations. Genes that were usually observed to be up-regulated in glucose-treated plants, were induced in the absence of glucose in RIL310, confirming the visual senescence phenotype.

6.3 Relationship between the regulation of senescence and flowering

In the Bay-0 x Shahdara collection, senescence appears to be dependent on flowering in that most early flowering lines are early senescing and vice versa. The slower flowering development of the male parent Shahdara, compared to Bay-0 (and some of the other RILs) was a trend seen throughout all the trials. This would not be unusual except that Shahdara has been reported to be an early flowering accession in a number of studies (Werner et al. 2005; Luquez et al. 2006). This implies that flowering in Shahdara is dependent on nitrogen status, as in both LN and LNG flowering was slower than most of the other RILs. An interaction between senescence and flowering is particularly evident in RIL 310 in which flowering is severely repressed and, although the oldest leaves senesce, young leaves continue to be produced at the top of the rosette. This phenotype does not hold true, however, if RIL 310 plants are exposed to a vernalisation treatment. In this case, the plants flower early and senescence quickly follows (A. Wingler, personal communication), suggesting that floral initiation triggers senescence. Other lines of evidence also suggest that senescence is related to floral initiation in Arabidopsis. Whereas removal of the inflorescence does not delay senescence of individual leaves (Hensel et al. 1993; Noodén and Penney 2001), flowering time correlates with leaf senescence in different Arabidopsis accessions (Levey and Wingler 2005). This shows that senescence in Arabidopsis is probably not regulated by fruit development, but by earlier processes, such as floral initiation. The results presented in this thesis show that sugar and nitrogen supply regulate flowering and could thereby also affect senescence. The promotion of flowering by sugars in Arabidopsis has been demonstrated before

(Roldán et al. 1999). Sugars could therefore provide a possible link between flowering and senescence, although the causal relationship has yet to be resolved.

6.4 QTL analysis of sugar-induced senescence

The QTL analysis revealed four QTL. The QTL discovered on Chromosomes II and IV were consistently detected and closely clustered, to indicate the presence of loci for glucose-induced senescence. The use of NILs successfully confirmed the QTL on chromosome IV as differences in the F_v/F_m phenotype of NILs 312 Bay-0 and 312 Shahdara were observed. In addition to the F_v/F_m, the flowering phenotype of the two pairs of NILs was different in that the Shahdara allele conveyed delayed reproductive development and, in the majority of cases, none of the plants flowered.
Only one pair of the NILs confirmed the QTL on chromosome IV. This is probably because the locus on chromosome IV interacts with at least one other locus to determine the senescence phenotype. Evidence for this was found from the ten latest senescing lines all of which had the Shahdara allele at the top of Chromosome IV. However, as all of these lines exhibited a more delayed phenotype than Shahdara there must be a second locus interacting with the locus on chromosome IV. NILs 145 Bay-0 and 145 Shahdara must have different alleles present in the second locus (or loci) that do not exert this interactive effect on the trait (high F_v/F_m).

Four QTL were detected in this study, two of which were consistently detected. Loudet et al. (2003) found between four and nine QTL involved in amino acid accumulation and nitrogen use, Diaz et al. (2006) found nine QLT involved in

yellowing and four for reddening and Luquez et al. (2006) found three QTL for post bolting longevity in LN conditions and one in high nitrogen. The number of QTL found in the present study was therefore similar. The three aforementioned studies are all relevant to the study presented herein and indeed some of the QTL located over lapped with those identified by Loudet et al (2002a) and, particularly, Diaz et al. (2006). This latter study is particularly relevant to senescence explaining the overlap. That the allelic effect of most of the authors' QTL was less than those for glucose induced senescence is perhaps evidence that glucose-dependent regulation occurs up-stream of other pathways regulating senescence. Thereby, sugars could act as integrators of several senescence pathways, e.g. regulated by nitrogen supply, light or stress, as discussed in Wingler et al. (2005).

Diaz et al. (2006) also used low nitrogen nutrition and plants were measured at DAP 35, but the group's plants were grown in compost as opposed to petri-dishes. Compost-grown plants live for considerably longer than those grown in sterile media and inevitably the two experiments would have experienced different environmental factors that must affect the comparability of the two experiments. A main difference between the results presented here and the QTL analysis by Diaz et al. (2006) is that the F_v/F_m values obtained in this thesis reflect whole-rosette senescence, whereas senescence of the first six leaves was analysed by Diaz and co-workers. These factors make it possible that the QTL detected by Diaz et al. (2006) and this study, relate to different genes of interest.

As whole-rosette senescence, as studied here, is related to flowering, the finding that the additional QTL found here, but not by Diaz et al. (2006) co-localises with the

flowering regulator *FRI* is very interesting. A possible function of *FRI* in the regulation of senescence is also suggested by the vernalisation-dependent regulation of flowering and senescence in RIL 310 as mentioned above.

Although cold acclimation and vernalisation are considered to be regulated by separate pathways, a gene involved in cold acclimation, *SFR6*, was also considered as candidate gene. The up-regulated expression of the cold tolerance gene *COR15b* in LNG presented some intriguing possibilities about the role of sugars in regulating cold tolerance. Cold-acclimated plants accumulate sugars but do not show the accelerated senescence phenotype usually associated with high leaf sugar contents (Masclaux-Daubresse et al. 2007). The greatest up-regulation compared to LN treatment was observed in RIL 310. This line also shows a delayed senescence phenotype when grown on LNG. It is therefore possible that the induction of cold tolerance genes by glucose in this line was responsible for its reduced response to glucose compared to the other RILs. If this hypothesis is correct the cold responsive gene governing this trait (not necessarily *COR15b*) could be responsible for the producing the variation in the timing of glucose-induced senescence, i.e. be the gene of interest.

The cold tolerance gene *SFR6* was also found to map very close to the QTL on chromosome IV and so a mutant plant was used to test response to treatment with glucose. Unfortunately, the degree of variation exhibited by *sfr6* mutant plants meant that no conclusions could be drawn and the F_v/F_m values between the mutant and wild type Col-0 were similar.

The candidate genes proposed in Chapter 5 all have properties relevant to senescence. The active location of *FRIGIDA* and *GLB1* is depicted in Figures 6.1 and 6.2. The sequencing of the *SFR6* gene is currently underway so if polymorphisms between this gene in Bay-0 and Shahdara exist, further experiments will be carried out to confirm whether this gene could be responsible for controlling the trait. A second micro-array study into glucose-induced senescence at a later time point (such as DAP 35), would also offer an insight into whether the nominated genes are differentially regulated by glucose treatment in the five characterised RILs.

6.5 Conclusion

The results presented in this study link the accumulation of sugars with the onset of senescence though the action of a genetic locus situated on chromosome four. It has also become clear that sugars interact with nitrogen and these two essential nutrients, in unison, can signal the nutritional status of the plant and adjust gene action accordingly. A strong relationship between whole rosette senescence and floral initiation has also been made apparent by the study of the Bay-0 x Shahdara population. Of particular interest and basis for further study is the identification of *FRIGIDA* as a candidate gene and that glucose may instigate senescence through regulation of this gene. The work presented in this thesis also suggests interactions of the metabolic regulation of leaf senescence with the regulation of flowering and cold

acclimation (Fig 6.3). Ultimately, this work has contributed to the current understanding of the complex yet elegant processes that comprise leaf senescence.

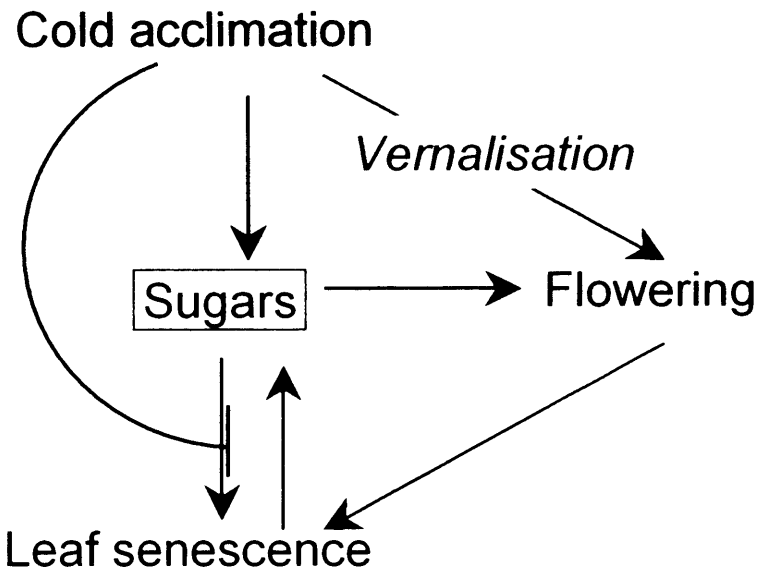


Figure 6.1 Model for the relationship between sugar accumulation, senescence, flowering and cold acclimation.

Cold acclimation causes an accumulation of leaf sugars that may then act as a signal to initiate flowering. Similarly, vernalisation can trigger reproductive development. A link between flowering and senescence has been proposed (Levey and Wingler, 2005) and elevated levels of sugar trigger leaf senescence.

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